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(54) Title: PLANT CELLS AND PLANTS WHICH SYNTHESIZE A STARCH WITH AN INCREASED FINAL VISCOSITY

(57) Abstract: The present invention relates to a plant cell which is genetically modified, the genetic modification leading to the reduction of the activity of one or more SSIII proteins which occur endogenously in the plant cell and to the reduction of the activity of one or more BEI proteins which occur endogenously in the plant cell and to the reduction of the activity of one or more BEII proteins which occur endogenously in the plant cell in comparison with corresponding plant cells, of wild-type plants, which have not been genetically modified. Further aspects of the invention relate to plants containing such plant cells, to a method for generating the plant cells and plants, and to the starch obtainable from them.

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**Plant cells and plants which synthesize a starch with an increased final viscosity**

## **5 Description**

The present invention relates to plant cells and plants which are genetically modified, the genetic modification leading to the reduction of the activity of SSIII and BEI and BEII proteins in comparison with  
10 corresponding plant cells, of wild-type plants, which have not been genetically modified. Furthermore, the present invention relates to means and methods for the generation of such plant cells and plants. Such plant cells and plants synthesize a modified starch which is characterized in that it has an amylose content of at least 30% and a phosphate content which  
15 is increased in comparison with starch from corresponding wild-type plants which have not been genetically modified and which have a final viscosity in the RVA analysis which is increased over the prior art and/or a modified side-chain distribution and/or an increased gel strength in the Texture Analyser and/or a modified granule morphology and/or a modified mean  
20 granule size. The present invention thus also relates to the starch synthesized by the plant cells and plants according to the invention, and to methods for producing this starch.

In view of the increasing importance which is currently attached to plant  
25 constituents as renewable raw materials, one of the tasks of biotechnology research is to attempt an adaptation of these vegetable raw materials to the requirements of the processing industry. In order to make possible the use of renewable raw materials in as many fields of application as possible, it is additionally necessary to arrive at very diverse substances.

The polysaccharide starch is a polymer of chemically uniform units, the glucose molecules. However, it takes the form of a highly complex mixture of different forms of molecules which differ with regard to their degree of polymerization and the occurrence of branches of the glucose chains.

- 5 Starch is therefore no uniform raw material. One distinguishes between two chemically different components of starch, amylose and amylopectin. In typical plants used for starch production such as, for example, maize, wheat or potato, amylose starch accounts for approximately 20%-30% and amylopectin starch for approximately 70%-80% of the starch synthesized.
- 10 Amylose has long been regarded as a linear polymer consisting of  $\alpha$ -1,4-glycosidically linked  $\alpha$ -D-glucose monomers. However, more recent studies have demonstrated the presence of  $\alpha$ -1,6-glycosidic branch points (approx. 0.1%) (Hizukuri and Takagi, Carbohydr. Res. 134, (1984), 1-10; Takeda et al., Carbohydr. Res. 132, (1984), 83-92).
- 15 Various methods are available for determining the amylose content. Some of these methods are based on the iodine-binding capacity of amylose, which can determine potentiometrically (Banks & Greenwood, in W. Banks & C.T. Greenwood, Starch and its components (pp. 51-66), Edinburgh, 20 Edinburgh University Press), amperometrically (Larson et al., Analytical Chemistry 25(5), (1953), 802-804) or spectrophotometrically (Morrison & Laignelet, J. Cereal Sc. 1, (1983), 9-20). The amylose content may also be determined calorimetrically by means of DSC (differential scanning calorimetry) measurements (Kugimiya & Donovan, Journal of Food 25 Science 46, (1981), 765-770; Sievert & Holm, Starch/Stärke 45 (4), (1993), 136-139). It is furthermore possible to determine the amylose content via the use of SEC (size exclusion chromatography) chromatography of native or debranched starch. This method has been recommended in particular for determining the amylose content of genetically modified starches 30 (Gérard et al., Carbohydrate Polymers 44, (2001), 19-27).

In contrast to amylose, amylopectin shows a higher degree of branching and has approximately 4% of branch points brought about by the occurrence of additional  $\alpha$ -1,6-glycosidic linkages. Amylopectin constitutes a complex mixture of glucose chains with different branching patterns.

- 5 Another important difference between amylose and amylopectin is their molecular weight. While amylose, depending on the origin of the starch, has a molecular weight of  $5 \times 10^5 - 10^6$  Da, the molecular weight of amylopectin is between  $10^7$  and  $10^8$  Da. The two macromolecules can be distinguished on the basis of their molecular weight and their different  
10 physico-chemical properties, and the simplest way of visualizing this is through their different iodine-binding properties.

- In addition to the amylose/amylopectin ratio and the phosphate content, the functional properties of starch are affected greatly by the molecular  
15 weight, the side-chain distribution pattern, the ionic content, the lipid and protein content, the mean granule size and the granule morphology and the like. Important functional properties which may be mentioned in this context are solubility, the retrogradation behaviour, the water-binding capacity, the film-forming properties, viscosity, the gelatinization  
20 properties, freeze-thaw-stability, acid stability, gel strength and the like. Granule size may also be of importance for various applications.

- The skilled workers frequently resort to different methods to determine the gelatinization properties, one of which is the final viscosity. Depending on  
25 the method used, absolute values in particular, but also relative values, may differ between one and the same starch sample. A rapid and effective method for analysing the gelatinization properties is the RVA analysis. Depending on the choice of the parameters and the temperature profile in the RVA analysis, different RVA profiles are obtained for one and the  
30 same sample. It should be mentioned that in some cases different profiles

were used in the prior art mentioned hereinbelow when determining the gelatinization properties.

An overview over different plant species with a reduction of the enzymes  
5 participating in starch biosynthesis can be found in Kossmann and Lloyd  
(2000, Critical Reviews in Plant Sciences 19(3), 171-126).

To date, plants have been described in which the activity of an SSIII  
protein (Abel et al., 1996, The Plant Journal 10(6), 9891-991; Lloyd et al.,  
1999, Biochemical Journal 338, 515-521) or the activity of a BEI protein  
10 (Kossmann et al. 1991, Mol Gen Genet 230, 39-44); Safford et al., 1998,  
Carbohydrate Polymers 35, 155-168, or the activity of a BEII protein  
(Jobling et al., 1999, The Plant Journal 18, or the activity of a BEI and a  
BEII protein (Schwall et al., 2000, Nature Biotechnology 18, 551- 554; WO  
96/34968), or the activity of a BEI and an SSIII (WO 00/08184) protein are  
15 reduced .

In plants in which the activity of an SSIII protein is reduced, a relative shift  
of the amylopectin side chains from longer chains towards shorter chains  
(Lloyd et al., 1999, Biochemical Journal 338, 515-521), a 70% higher  
phosphate content, no changes in the amylose content (Abel et al., 1996,  
20 The Plant Journal 10(6), 9891-991) and a reduced final viscosity in the  
RVA analysis (Abel, 1995, PhD Thesis at the Freie Universität Berlin) are  
observed in comparison with corresponding wild-type plants. In such  
plants, which are also described in WO 00/08184, a 197% increase in the  
phosphate content, 123% increase in the amylose content and a final  
25 viscosity in the RVA analysis which drops to 76% of that of the wild type  
can be observed in the isolated starch in comparison with untransformed  
wild-type plants. Moreover, the gel strength of the starch in question drops  
to 84% of the wild type.

30 The spectrophotometric analysis by the method of Morrison & Laignelet  
(1983, J. Cereal Sc. 1, 9-20) reveals an amylose content of up to a

maximum of 89.14% (corresponding to 344% of the wild type) and a starch phosphate content which corresponds to up to a maximum of 522% of the phosphate content of starch isolated from corresponding wild-type plants in plants with a reduced activity of both a BEI and a BEII protein. The RVA  
5 analysis reveals a final viscosity value in these starches which is increased up to a maximum of 237%. Moreover, the modified granule morphology in starch grains isolated from such plants is distinguished by the fact that the granules have large grooves in the centre of the granule in question when viewed under the microscope under polarized light.

10

As a result, the skilled worker is familiar with plant cells and plants and starches synthesized by them which have an increased amylose and phosphate content but whose final viscosity in the RVA analysis is increased by not more than up to a maximum of 256% in comparison with  
15 wild-type plants which have not been genetically modified. Higher final viscosities in the RVA analysis have not been achieved to date. However, this would be desirable since less starch solids have to be employed, for example when using such a starch as thickener, gelling agent or binder, in order to achieve the desired effect. This allows for example the reduction  
20 of the amount of additives in human and animal foods, in healthcare products and in cosmetics. It would also be possible to employ smaller amounts of starch when using such a starch in glues, leading to reduced costs for example in making, for example, paper, cardboard and insulating board.

25 The present invention is thus based on the object of providing plant cells, plants and starch from suitable plant cells or plants with an increased amylose content and an increased phosphate content and, in the RVA analysis, a final viscosity which is increased by at least 270% and/or an increased gel strength of the gelatinized starch and/or a modified granule  
30 morphology.

This object is achieved by providing the embodiments specified in the patent claims.

A first aspect of the present invention thus relates to a plant cell which is  
5 genetically modified, the genetic modification leading to the reduction of  
the activity of one or more SSIII proteins occurring endogenously in the  
plant cell and to the reduction of the activity of one or more BEI proteins  
which occur endogenously in the plant cell and to the reduction of the  
activity of one or more BEII proteins which occur endogenously in the plant  
10 cell in comparison to corresponding plant cells, of wild-type plants, which  
have not been genetically modified.

In this context, the genetic modification can be any genetic modification  
which leads to a reduction of the activity of one or more SSIII proteins  
15 which occur endogenously in the plant cell and to the reduction of the  
activity of one or more BEI proteins which occur endogenously in the plant  
cell and to the reduction of the activity of one or more BEII proteins which  
occur endogenously in the plant cell in comparison to corresponding plant  
cells, of wild-type plants, which have not been genetically modified.

20

For the purposes of the invention, the genetic modification may  
encompass for example the generation of plant cells according to the  
invention by subjecting one or more genes to mutagenesis. The type of  
mutation is irrelevant as long as it leads to a reduction of the activity of an  
25 SSIII protein and/or a BEI protein and/or a BEII protein. In connection with  
the present invention, the term "mutagenesis" is understood as meaning  
any type of mutation, such as, for example, deletions, point mutations  
(nucleotide substitutions), insertions, inversions, gene conversions or  
chromosome translocation.

30

In this context, the mutation can be generated by using chemical agents or high-energy radiation (for example x-rays, neutron, gamma, UV radiation). Agents which can be employed for generating chemically induced mutations, and the mutations generated thereby by the action of the mutagens in question, are described, for example, by Ehrenberg and Husain, 1981, (Mutation Research 86, 1-113), Müller, 1972 (Biologisches Zentralblatt 91 (1), 31-48). The generation of rice mutants using gamma rays, ethyl methanesulfonate (EMS), N-methyl-N-nitrosurea or sodium azide (NaN<sub>3</sub>) is described, for example, in Jauhar and Siddiq (1999, Indian Journal of Genetics, 59 (1), 23-28), in Rao (1977, Cytologica 42, 443-450), Gupta and Sharma (1990, Oryza 27, 217-219) and Satoh and Omura (1981, Japanese Journal of Breeding 31 (3), 316-326). The generation of wheat mutants using NaN<sub>3</sub> or maleic hydrazide is described in Arora et al. (1992, Anals of Biology 8 (1), 65-69). An overview over the generation of wheat mutants using different types of high-energy radiation and chemical agents is given in Scarascia-Mugnozza et al. (1993, Mutation Breeding Review 10, 1-28). Svec et al. (1998, Cereal Research Communications 26 (4), 391-396) describes the use of N-ethyl-N-nitrosurea for generating mutants in triticale. The use of MMS and gamma radiation for generating millet mutants is described in Shashidhara et al. (1990, Journal of Maharashtra Agricultural Universities 15 (1), 20-23).

The generation of mutants in plant species whose propagation is predominantly vegetatively was described for example for potatoes which produce a modified starch (Hovenkamp-Hermelink et al. (1987, Theoretical and Applied Genetics 75, 217-221) and for mint with an increased oil yield/modified oil quality (Dwivedi et al., 2000, Journal of Medicinal and Aromatic Plant Sciences 22, 460-463). All of these methods are suitable in principle for generating the plant cells according to the invention and the starch produced by them.



Mutations in the relevant genes, in particular in genes encoding a BEI protein and/or a BEII protein and/or an SSIII protein, can be identified with the aid of methods known to the skilled worker. Analyses based on hybridizations with probes (Southern blot), the amplification by means of  
5 polymerase chain reaction (PCR), the sequencing of genomic sequences in question, and the search for individual nucleotide substitutions, may be employed in particular. One method of identifying mutations with the aid of hybridization patterns is, for example, the search for restriction fragment length polymorphisms (RFLP) (Nam et al., 1989, *The Plant Cell* 1, 699-  
10 705; Leister and Dean, 1993, *The Plant Journal* 4 (4), 745-750). An example of a method based on PCR is the analysis of amplified fragment length polymorphisms (AFLP) (Castiglioni et al., 1998, *Genetics* 149, 2039-2056; Meksem et al., 2001, *Molecular Genetics and Genomics* 265, 207-214; Meyer et al., 1998, *Molecular and General Genetics* 259, 150-  
15 160). The use of amplified fragments cleaved with the aid of restriction endonucleases (cleaved amplified polymorphic sequences, CAPS) may also be used for identifying mutations (Konieczny and Ausubel, 1993, *The Plant Journal* 4, 403-410; Jarvis et al., 1994, *Plant Molecular Biology* 24, 685-687; Bachem et al., 1996, *The Plant Journal* 9 (5), 745-753). Methods  
20 for determining SNPs have been described, inter alia, by Qi et al. (2001, *Nucleic Acids Research* 29 (22), e116) Drenkard et al. (2000, *Plant Physiology* 124, 1483-1492) and Cho et al. (1999, *Nature Genetics* 23, 203-207). Methods which are particularly suitable are those which permit a large number of plants to be analysed within a short time for mutations in  
25 specific genes. Such a method, known as TILLING (targeting induced local lesions in genomes), has been described by McCallum et al. (2000, *Plant Physiology* 123, 439-442).

The use of all of these methods is suitable in principle for the purposes of  
30 the present invention.

Hoogkamp et al. (2000, Potato Research 43, 179-189) have isolated stable potato mutants which contain an amylose-free starch. These plants no longer synthesize active enzyme for a granule-bound starch synthase (GBSS I). After subjecting these plants to another mutagenesis, those  
5 which additionally have mutations in genes which are involved in starch biosynthesis may be selected. Plants which synthesize starch with improved characteristics might thus be generated. Using the suitable method, it is also possible to identify and isolate the plant cells according to the invention which produce a starch according to the invention.

10

Moreover, the plant cells according to the invention may also be generated with the aid of homologous transposons, that is to say transposons which are naturally present in the plant cells in question. A detailed description of this method is given hereinbelow.

15

All of the abovementioned methods are suitable in principle for generating plant cells according to the invention and the modified starch synthesized by them. The present invention therefore also relates to methods for generating genetically modified plant cells which synthesize a modified  
20 starch, this starch being characterized in that it has an amylose content of at least 30%, in that it has an increased phosphate content in comparison with starch from corresponding wild-type plant cells which have not been genetically modified and in that it has an increased final viscosity in the RVA analysis in comparison with starch from corresponding wild-type plant  
25 cells which have not been genetically modified.

A further aspect of the present invention relates to methods for generating  
a plant cell which synthesizes a modified starch, comprising the genetic  
modification of the plant cell, the genetic modification leading to the  
30 reduction of the activity of one or more SSIII proteins which occur endogenously in the plant cell and to the reduction of the activity of one or

more BEI proteins which occur endogenously in the plant cell and to the reduction of the activity of one or more BEII proteins which occur endogenously in the plant cell, in comparison with corresponding plant cells, of wild-type plants, which have not been genetically modified.

5

Yet a further aspect of the present invention relates to methods for generating a genetically modified plant which synthesizes a modified starch, in which

- a) a plant cell is generated as described above;
- 10 b) a plant is regenerated from, or using, the plant cell generated in accordance with a); and,
- c) if appropriate, further plants are generated from the plant generated in accordance with step b).

- 15 In connection with the present invention, the term "genetically modified" means that the genetic information of the plant cell is altered.

In this context, a reduction of the activity of one or more SSIII proteins which occur endogenously in the plant cell and a reduction of the activity  
20 of one or more BEI proteins which occur endogenously in the plant cell and a reduction of the activity of one or more BEII proteins which occur endogenously in the plant cell is observed in the plant cells according to the invention in comparison with corresponding plant cells, of wild-type plants, which have not been genetically modified.

25

The genetic modifications for generating the plant cells according to the invention can be performed simultaneously or in consecutive steps. In this context, each genetic modification can lead to the reduction of the activity  
30 of one or more SSIII proteins and/or one or more BEI proteins and/or one or more BEII proteins. The starting material may be either wild-type plants or wild-type plant cells in which no previous genetic modification in order to

reduce the activity of one or more SSIII proteins and/or one or more BEI proteins and/or one or more BEII proteins has been performed, or else genetically modified plant cells or plants in which the activity of one or more SSIII proteins and/or one or more BEI proteins and/or one or more BEII proteins has already been carried out by genetic modification. If such

5 genetically modified plants (plant cells) constitute the starting material, the genetic modifications which are subsequently carried out preferably only relate to the activity of in each case one or more proteins whose activity has not been reduced yet (SSIII, BEI or BEII).

10

For example, a reduction of the expression of one or more SSIII genes which occur endogenously in the plant cell and a reduction of the expression of one or more BEI genes which occur endogenously in the plant cell and a reduction of the expression of one or more BEII genes

15 which occur endogenously in the plant cell and/or a reduction of the activity of in each case one or more of the abovementioned proteins which occur in the plant cell is observed in genetically modified plant cells according to the invention in comparison with plant cells, of wild-type plants, which have not been genetically modified.

20

For the purposes of the present invention, the term "reduction of the activity" refers to a reduction of the expression of endogenous genes which encode SSIII, BEI and/or BEII proteins, and/or a reduction of the amount of SSIII, BEI and/or BEII protein in the cells and/or a reduction of

25 the enzymatic activity of the SSIII, BEI and/or BEII proteins in the cells.

The reduction of the expression can be determined for example by measuring the amount of SSIII, BEI or BEII protein-encoding transcripts, for example by Northern blot analysis or RT-PCR. A reduction preferably

30 means, in this context, a reduction of the amount of transcripts by at least 50%, in particular by at least 70%, preferably by at least 85% and

especially preferably by at least 95% in comparison to corresponding cells which have not been genetically modified.

The reduction of the amount of SSIII, BEI and/or BEII proteins which results in a reduced activity of these proteins in the plant cells in question  
5 can be determined for example by immunological methods such as Western blot analysis, ELISA (enzyme-linked immunosorbent assay) or RIA (radio-immune assay). In this context, a reduction preferably means a reduction of the amount of SSIII, BEI and/or BEII protein by at least 50%, in particular by at least 70%, preferably by at least 85% and especially  
10 preferably by at least 95% in comparison to corresponding cells which have not been genetically modified.

In connection with the present invention, SSIII protein is understood as meaning a class of soluble starch synthases (ADP-glucose-1,4-alpha-D-  
15 glucan-4-alpha-D-glucosyltransferase; EC 2.4.1.21). Soluble starch synthases catalyze a glycosylation reaction, in which glucose residues of the substrate ADP-glucose are transferred to alpha-1,4-linked glucan chains, with formation of an alpha-1,4 linkage ( $\text{ADP glucose} + \{(1,4)\text{-alpha-D-glucosyl}\}(N) \rightleftharpoons \text{ADP} + \{(1,4)\text{-alpha-D-glucosyl}\}(N+1)$ ).

20

SSIII proteins are described, for example, by Marshall et al. (The Plant Cell 8; (1996); 1121-1135), Li et al. (2000, Plant Physiology 123, 613-624), Abel et al. (The Plant Journal 10(6); (1996); 981-991) and in WO 0066745. The structure of SSIII proteins frequently shows a sequence of domains.

25 At the N terminus, SSIII proteins have a signal peptide for the transport into plastids. Towards the C terminus, this is followed by an N-terminal region, an SSIII-specific region and a catalytic domain (Li et al., 2000, Plant Physiology 123, 613-624). Further analyses which are based on primary sequence alignments (<http://hits.isb-sib.ch/cgi-bin/PFSCAN>),  
30 revealed that the potato SSIII protein has what is known as a carbohydrate binding domain (CBM). This domain (Pfam motif cbm 25) comprises the

amino acids 377 to 437 of the sequence of the potato SSIII protein shown in Seq ID No. 2. In connection with the present invention, an SSIII protein is therefore to be understood as meaning starch synthases which have at least 50%, preferably at least 60%, especially preferably at least 70%,  
5 more preferably at least 80% and in particular at least 90% identity with the sequence shown in Seq ID No. 3.

The term homology, or identity, is understood as meaning the number of agreeing amino acids (identity) with other proteins, expressed in percent.  
10 The identity is preferably determined by comparing the Seq. ID No. 3 with other proteins with the aid of computer programmes. If sequences which are compared with each other are different in length, the identity is to be determined in such a way that the number of amino acids which the short sequence shares with the longer sequence determines the percentage  
15 identity. The identity can be determined routinely by means of known computer programmes which are publicly available such as, for example, ClustalW (Thompson et al., Nucleic Acids Research 22 (1994), 4673-4680). ClustalW is made publicly available by Julie Thompson (Thompson@EMBL-Heidelberg.DE) and Toby Gibson (Gibson@EMBL-  
20 Heidelberg.DE), European Molecular Biology Laboratory, Meyerhofstrasse 1, D 69117 Heidelberg, Germany. ClustalW can likewise be downloaded from various internet pages, inter alia the IGBMC (Institut de Génétique et de Biologie Moléculaire et Cellulaire, B.P.163, 67404 Illkirch Cedex, France; <ftp://ftp-igbmc.u-strasbg.fr/pub/>) and the EBI  
25 (<ftp://ftp.ebi.ac.uk/pub/software/>) and all mirrored EBI internet pages (European Bioinformatics Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SD, UK).

If the ClustalW computer programme Version 1.8 is used to determine the identity between, for example, the reference protein of the present  
30 application and other proteins, the following parameters are to be set: KTUPLE=1, TOPDIAG=5, WINDOW=5, PAIRGAP=3, GAOPEN=10,

GAPEXTEND=0.05, GAPDIST=8, MAXDIV=40, MATRIX=GONNET, ENDGAPS(OFF), NOPGAP, NOHGAP.

One possibility of finding similar sequences is to carry out sequence database researches. Here, one or more sequences are entered as what  
5 is known as a query. This query sequence is then compared with sequences present in the selected databases using statistical computer programmes. Such database queries (blast searches) are known to the skilled worker and can be carried out at different suppliers. If, for example, such a database query is carried out at the NCBI (National Center for  
10 Biotechnology Information, <http://www.ncbi.nlm.nih.gov/>), the standard settings for the respective comparison query should be used. For protein sequence comparisons (blastp), these settings are: Limit entrez = not activated; Filter = low complexity activated; Expect value = 10; word size = 3; Matrix = BLOSUM62; Gap costs: Existence = 11, Extension = 1. The  
15 result of such a query is, among other parameters, the degree of identity between the query sequence and the similar sequences found in the databases.

Thus, an SSIII protein is to be understood as meaning, in connection with the present invention, starch synthases which, when using at least one of  
20 the above-described methods for determining the identity with the sequence shown in Seq ID No. 3, have at least 50%, preferably at least 60%, especially preferably at least 70%, more preferably at least 80% and in particular at least 90% identity.

25 For the purposes of the present invention, the term SSIII gene is understood as meaning a nucleic acid molecule (DNA, cDNA, RNA) which encodes an SSIII protein, preferably from potato. Nucleic acid molecules encoding an SSIII protein have been described for a variety of plant species such as, for example, potato (Abel et al., The Plant Journal 10(6);  
30 (1996); 981-991), wheat (WO 00/66745, Li et al., 2000, Plant Physiology 123, 613-624; Genbank Acc. No AF258608; Genbank Acc. No

AF258609), maize (Gao et al., 1998, Plant Cell 10 (3), 399-412; Genbank Acc. No AF023159), *Vigna* (Genbank Acc. No AJ225088), rice (Genbank Acc. No AY100469; Genbank Acc. No AF43291) and *Arabidopsis* (Genbank Acc. No AC007296).

5

For the purposes of the present invention, the term "branching enzyme" or "BE protein" ( $\alpha$ -1,4-glucan:  $\alpha$ -1,4-glucan-6-glycosyltransferase, E.C. 2.4.1.18) is understood as meaning a protein which catalyzes a transglycosylation reaction in which  $\alpha$ -1,4-linkages of an  $\alpha$ -1,4-glucan donor are hydrolyzed and the  $\alpha$ -1,4-glucan chains liberated in this process are transferred to an  $\alpha$ -1,4-glucan acceptor chain, where they are converted into  $\alpha$ -1,6 linkages.

The term "BEI protein" is to be understood as meaning, for the purposes of the present invention, an isoform I branching enzyme (branching enzyme = BE). The BEI protein is preferably derived from potato plants.

In this context, isoform terminology relies on the nomenclature proposed by Smith-White and Preiss (Smith-White and Preiss, Plant Mol Biol. Rep. 12, (1994), 67-71, Larsson et al., Plant Mol Biol. 37, (1998), 505-511). This nomenclature assumes that all enzymes which have a higher degree of homology (identity) at the amino acid level with the maize BEI protein (GenBank Acc. No. D11081; Baba et al., Biochem. Biophys. Res. Commun. 181 (1), (1991), 87-94; Kim et al. Gene 216, (1998), 233-243) than to the maize BEII protein (Genbank Acc. No AF072725 , U65948) are referred to as isoform I branching enzymes, abbreviated to BEI proteins.

The term "BEII protein" is to be understood as meaning, for the purposes of the present invention, an isoform II branching enzyme (branching enzyme = BE). This enzyme preferably originates from potato plants. In connection with the present invention, all enzymes which, at the amino acid level, have a higher degree of homology (identity) with the maize BEII



protein (Genbank Acc. No AF072725, U65948) than with the maize BEI protein (Genbank Acc. No. D 11081, AF 072724 ) shall be referred to as BEII protein.

- 5 The term "BEI gene" is understood as meaning, for the purposes of the present invention, a nucleic acid molecule (cDNA, DNA) which encodes a "BEI protein", preferably a BEI protein from potato plants. Such nucleic acid molecules have been described for a large number of plants, for example for maize (Genbank Acc. No. D 11081, AF 072724), rice  
10 (Genbank Acc. No. D11082), pea (Genbank Acc. No. X80010) and potato. Various forms of the BEI gene, or the BEI protein, from potato have been described, for example, by Khoshnoodi et al., Eur. J. Biochem. 242 (1), 148-155 (1996), Genbank Acc. No. Y 08786 and by Kossmann et al., Mol. Gen. Genet. 230, (1991), 39-44). In potato plants, the BEI gene is  
15 expressed predominantly in the tubers and to a very minor degree in the leaves (Larsson et al., Plant Mol. Biol. 37, (1998), 505-511).

- The term "BEII gene" is to be understood as meaning, for the purposes of the present invention, a nucleic acid molecule (for example cDNA, DNA)  
20 which encodes a "BEII protein", preferably a BEII protein from potato plants. Such nucleic acid molecules have been described for a large number of plants, for example for potato (GenBank Acc. No. AJ000004, AJ011888, AJ011889, AJ011885, AJ011890, EMBL GenBank A58164), maize (AF 072725, U65948), barley (AF064561), rice (D16201) and wheat  
25 (AF 286319). In potato plants, the BEII gene is expressed predominantly in the tubers and to a very minor degree in the leaves (Larsson et al., Plant Mol. Biol. 37, (1998), 505-511).

- The term "transgenic" is to be understood as meaning, in the present  
30 context, that the genetic information of the plant cells according to the invention deviates from corresponding plant cells which have not been

genetically modified owing to the introduction of a foreign nucleic acid molecule or several foreign nucleic acid molecules into the cell.

In a further embodiment of the present invention, the genetic modification  
5 of the transgenic plant cell according to the invention consists in the introduction of one or more foreign nucleic acid molecules whose presence and/or expression leads to the reduction of the activity of SSIII and BEI and BEII proteins in comparison to corresponding plant cells, of wild-type plants, which have not been genetically modified. Specifically,  
10 the term "genetic manipulation" is understood as meaning the introduction of homologous and/or heterologous nucleic acid molecules and/or foreign nucleic acid molecules which have been subjected to mutagenesis into a plant cell, where said introduction of these molecules leads to the reduction of the activity of an SSIII protein and/or a BEI protein and/or BEII  
15 protein.

The term "foreign nucleic acid molecule" or "of foreign nucleic acid molecules" is understood as meaning, for the purposes of the present invention, such a molecule which either does not occur naturally in the  
20 plant cells in question, or which does not naturally occur in the plant cells in the specific spatial arrangement, or which is localized at a site in the genome of the plant cell where it does not occur naturally. The foreign nucleic acid molecule is preferably a recombinant molecule which consists of various elements whose combination, or specific spatial arrangement,  
25 does not naturally occur in plant cells.

The foreign nucleic acid molecule(s) which is, or are, used for the genetic modification may take the form of a hybrid nucleic acid construct or of several separate nucleic acid constructs, in particular of what are known as single, dual and triple constructs. Thus, the foreign nucleic acid  
30 molecule may be, for example, what is known as a "triple construct", which is understood as meaning one single vector for plant transformation which

contains not only the genetic information for inhibiting the expression of one or more endogenous SSIII genes, but also the genetic information for inhibiting the expression of one or more BEI genes and of one or more BEII genes, or whose presence, or expression, leads to the reduction of the activity of one or more SSIII, BEI and BEII proteins.

In a further embodiment, the foreign nucleic acid molecule may be what is known as a "dual construct", which is understood as meaning a vector for plant transformation which contains the genetic information for inhibiting the expression of two out of the three target genes (SSIII, BEI, BEII gene) or whose presence, or expression, leads to the reduction of the activity of two out of the three target proteins (SSIII, BEI, BEII proteins). The inhibition of the expression of the third target gene and/or the reduction of the activity of the third target protein is effected, in this embodiment of the invention, with the aid of a separate, foreign nucleic acid molecule which contains the relevant genetic information for inhibiting this third target gene.

In a further embodiment of the invention, it is not a triple construct which is introduced into the genome of the plant cell, but several different foreign nucleic acid molecules are introduced, one of these foreign nucleic acid molecules being, for example, a DNA molecule which constitutes, for example, a cosuppression construct which brings about a reduction of the expression of one or more endogenous SSIII genes, and a further foreign nucleic acid molecule being a DNA molecule which encodes, for example, an antisense RNA which brings about a reduction of the expression of one or more endogenous BEI and/or BEII genes. When constructing the foreign nucleic acid molecules, however, the use of any combination of antisense, cosuppression, ribozyme and double-stranded RNA constructs or in-vivo mutagenesis which leads to a simultaneous reduction of the gene expression of endogenous genes encoding one or more SSIII, BEI

and BEII proteins, or which leads to a simultaneous reduction of the activity of one or more SSIII, BEI and BEII proteins, is also suitable in principle.

The foreign nucleic acid molecules can be introduced into the genome of  
5 the plant cell either simultaneously ("cotransformation") or else one after the other, i.e. in succession at different times ("supertransformation").

The foreign nucleic acid molecules can also be introduced into different individual plants of one species. This may give rise to plants in which the  
10 activity of one target protein, or two target proteins, (BEI, BEII, SSIII) is reduced. Subsequent hybridizing may then give rise to plants in which the activity of all three of the target proteins is reduced.

Instead of a wild-type plant cell or plant, a mutant which is distinguished by  
15 already showing a reduced activity of one or more target proteins (BEI, BEII, SSIII) may further be used for introducing a foreign nucleic acid molecule or for generating the plant cells or plants according to the invention. The mutants may take the form of spontaneously occurring mutants or else of mutants which have been generated by the specific  
20 application of mutagens. Possibilities of generating such mutants have been described further above.

The plant cells according to the invention and their starch can be generated, or produced, by using what is known as insertion mutagenesis  
25 (review article: Thorneycroft et al., 2001, Journal of experimental Botany 52 (361), 1593-1601). Insertion mutagenesis is to be understood as meaning, in particular, the insertion of transposons or what is known as transfer DNA (T-DNA) into a gene encoding a BEI protein and/or BEII protein and/or an SSIII protein, thus reducing the activity of said proteins in  
30 the cell in question.

The transposons may take the form of transposons which occur naturally in the cell (endogenous transposons) or else those which do not occur naturally in said cell but have been introduced into the cell by means of recombinant methods, such as, for example, by transforming the cell

5 (heterologous transposons). Modifying the expression of genes by means of transposons is known to the skilled worker. A review of the utilization of endogenous and heterologous transposons as tools in plant biotechnology can be found in Ramachandran and Sundaresan (2001, Plant Physiology and Biochemistry 39, 234-252). The possibility of identifying mutants in

10 which specific genes have been inactivated by transposon insertion mutagenesis can be found in a review by Maes et al. (1999, Trends in Plant Science 4 (3), 90-96). The generation of rice mutants with the aid of endogenous transposons is described by Hirochika (2001, Current Opinion in Plant Biology 4, 118-122). The identification of maize genes with the aid

15 of endogenous retrotransposons is shown, for example, in Hanley et al. (2000, The Plant Journal 22 (4), 557-566). The possibility of generating mutants with the aid of retrotransposons and methods for identifying mutants are described by Kumar and Hirochika (2001, Trends in Plant Science 6 (3), 127-134). The activity of heterologous transposons in

20 different species has been described both for dicotyledonous and for monocotyledonous plants, for example for rice (Greco et al., 2001, Plant Physiology 125, 1175-1177; Liu et al., 1999, Molecular and General Genetics 262, 413-420; Hiroyuki et al., 1999, The Plant Journal 19 (5), 605-613; Jeon and Gynheung, 2001, Plant Science 161, 211-219), barley

25 (2000, Koprek et al., The Plant Journal 24 (2), 253-263), *Arabidopsis thaliana* (Aarts et al., 1993, Nature 363, 715-717, Schmidt and Willmitzer, 1989, Molecular and General Genetics 220, 17-24; Altmann et al., 1992, Theoretical and Applied Genetics 84, 371-383; Tissier et al., 1999, The Plant Cell 11, 1841-1852), tomato (Belzile and Yoder, 1992, The Plant

30 Journal 2 (2), 173-179) and potato (Frey et al., 1989, Molecular and

General Genetics 217, 172-177; Knapp et al., 1988, Molecular and General Genetics 213, 285-290).

- In principle, the plant cells and plants according to the invention, and the starch produced by them, can be generated, or produced, with the aid of both homologous and heterologous transposons, the use of homologous transposons also including those transposons which are already naturally present in the plant genome.
- 10 T-DNA insertion mutagenesis is based on the fact that certain segments (T-DNA) of Ti plasmids from *Agrobacterium* are capable of integrating into the genome of plant cells. The site of integration into the plant chromosome is not fixed but may take place at any position. If the T-DNA integrates in a segment of the chromosome which constitutes a gene
- 15 function, this may lead to a modification of the gene expression and thus also to an altered activity of a protein encoded by the gene in question. In particular, the integration of a T-DNA into the coding region of a protein frequently means that the protein in question can no longer be synthesized in active form, or not at all, by the cell in question. The use of T-DNA
- 20 insertions for the generation of mutants is described, for example, for *Arabidopsis thaliana* (Krysan et al., 1999, The Plant Cell 11, 2283-2290; Atipiroz-Leehan and Feldmann, 1997, Trends in genetics 13 (4), 152-156; Parinov and Sundaresan, 2000, Current Opinion in Biotechnology 11, 157-161) and rice (Jeon and An, 2001, Plant Science 161, 211-219; Jeon et
- 25 al., 2000, The Plant Journal 22 (6), 561-570). Methods for identifying mutants which have been generated with the aid of T-DNA insertion mutagenesis are described, inter alia, by Young et al., (2001, Plant Physiology 125, 513-518); Parinov et al. (1999, The Plant cell 11, 2263-2270), Thorneycroft et al. (2001, Journal of Experimental Botany 52, 1593-
- 30 1601), and McKinney et al. (1995, The Plant Journal 8 (4), 613-622).

In principle, T-DNA mutagenesis is suitable for generating the plant cells according to the invention and for producing the starch produced by them.

In a further embodiment of the present invention, the presence and/or the  
5 expression of one or more foreign nucleic acid molecules leads to the inhibition of the expression of endogenous genes which encode SSIII proteins, BEI proteins and BEII proteins.

The plant cells according to the invention can be generated by various  
10 methods with which the skilled worker is familiar, for example by those which lead to an inhibition of the expression of endogenous genes encoding an SSIII, BEI or BEII protein. They include, for example, the expression of a corresponding antisense RNA or a double-stranded RNA construct, the provision of molecules or vectors which confer a  
15 cosuppression effect, the expression of a suitably constructed ribozyme which specifically cleaves transcripts encoding an SSIII, BEI or BEII protein, or what is known as "in-vivo mutagenesis". Moreover, the reduction of the SSIII and/or BEI and/or BEII activity in the plant cells may also be brought about by the simultaneous expression of sense and  
20 antisense RNA molecules of the specific target gene to be repressed, preferably the SSIII and/or BEI and/or BEII gene. The skilled worker is familiar with these methods.

Moreover, it is known that the generation *in planta* of double-stranded RNA molecules of promoter sequences *in trans* can lead to methylation  
25 and transcriptional inactivation of homologous copies of this promoter (Mette et al., EMBO J. 19, (2000), 5194-5201).

Other methods for reducing the activity of proteins are described hereinbelow.

All of these methods are based on the introduction of one or more foreign  
30 nucleic acid molecules into the genome of plant cells.

To inhibit gene expression by means of antisense or cosuppression technology it is possible to use, for example, a DNA molecule which encompasses all of the sequence encoding an SSIII and/or BEI and/or BEII protein including any flanking sequences which may be present, or  
5 else DNA molecules which only encompass parts of the coding sequence, which must be long enough in order to bring about an antisense effect, or cosuppression effect, in the cells. Sequences which are suitable generally have a minimum length of not less than 15 bp, preferably a length of 100-500 bp, and for effective antisense or cosuppression inhibition in particular  
10 sequences which have a length of over 500 bp.

Another possibility which is suitable for antisense or cosuppression approaches is the use of DNA sequences with a high degree of homology with the endogenous sequences which encode SSIII, BEI or BEII proteins  
15 and which occur endogenously in the plant cell. The minimum degree of homology should exceed approximately 65%. The use of sequences with homology levels of at least 90%, in particular between 95 and 100%, is to be preferred.

20 The use of introns, i.e. noncoding regions of genes, which encode SSIII, BEI and/or BEII proteins is also feasible for achieving an antisense or cosuppression effect.

The use of intron sequences for inhibiting the gene expression of genes which encode starch biosynthesis proteins has been described in the  
25 international patent applications WO97/04112, WO97/04113, WO98/37213, WO98/37214.

The skilled worker is familiar with methods for achieving an antisense and cosuppression effect. The cosuppression inhibition method has been described, for example, in Jorgensen (Trends Biotechnol. 8 (1990), 340-344), Niebel et al., (Curr. Top. Microbiol. Immunol. 197 (1995), 91-103),  
30 Flavell et al. (Curr. Top. Microbiol. Immunol. 197 (1995), 43-46), Palaqui



and Vaucheret (Plant. Mol. Biol. 29 (1995), 149-159), Vaucheret et al., (Mol. Gen. Genet. 248 (1995), 311-317), de Borne et al. (Mol. Gen. Genet. 243 (1994), 613-621).

- 5 The expression of ribozymes for reducing the activity of specific enzymes in cells is also known to the skilled worker and described, for example, in EP-B1 0321201. The expression of ribozymes in plant cells has been described, for example, in Feyter et al. (Mol. Gen. Genet. 250, (1996), 329-338).

10

Moreover, the reduction of the SSIII and/or BEI and/or BEII activity in the plant cells may also be achieved by what is known as "in-vivo mutagenesis", where an RNA-DNA oligonucleotide hybrid ("chimeroplast") is introduced into cells by means of transforming cells (Kipp, P.B. et al.,

- 15 Poster Session at the " 5<sup>th</sup> International Congress of Plant Molecular Biology, 21st-27th September 1997, Singapore; R. A. Dixon and C.J. Arntzen, Meeting report on "Metabolic Engineering in Transgenic Plants", Keystone Symposia, Copper Mountain, CO, USA, TIBTECH 15, (1997), 441-447; International Patent Application WO 9515972; Kren et al.,  
20 Hepatology 25, (1997), 1462-1468; Cole-Strauss et al., Science 273, (1996), 1386-1389; Beetham et al., 1999, PNAS 96, 8774-8778).

Part of the DNA component of the RNA-DNA oligonucleotide is homologous with a nucleic acid sequence of an endogenous SSIII, BEI and/or BEII gene, but contains a mutation in comparison with the nucleic  
25 acid sequence of an endogenous SSIII, BEI and/or BEII gene or contains a heterologous region which is surrounded by the homologous regions.

- Owing to base pairing of the homologous regions of the RNA-DNA oligonucleotide and of the endogenous nucleic acid molecule, followed by homologous recombination, the mutation or heterologous region contained  
30 in the DNA component of the RNA-DNA oligonucleotide can be transferred

into the genome of a plant cell. This leads to a reduction of the activity of one or more SSIII, BEI and/or BEII proteins.

Moreover, the reduction of the SSIII and/or BEI and/or BEII activity in the plant cells may also be caused by the simultaneous expression of sense  
5 and antisense RNA molecules of the specific target gene to be repressed, preferably the SSIII and/or BEI and/or BEII gene.

This may be achieved for example by the use of chimeric constructs which contain "inverted repeats" of the respective target gene or parts of the target gene. The chimeric constructs encode sense and antisense RNA  
10 molecules of the target gene in question. Sense and antisense RNA are synthesized simultaneously *in planta* as one RNA molecule, it being possible for sense and antisense RNA to be separated from each other by a spacer and to form a double-stranded RNA molecule.

It has been demonstrated that the introduction of inverted-repeat DNA  
15 constructs in the genome of plants is a highly effective method for repressing the genes corresponding to the inverted-repeat DNA constructs (Waterhouse et al., Proc. Natl. Acad. Sci. USA 95, (1998), 13959-13964; Wang and Waterhouse, Plant Mol. Biol. 43, (2000), 67-82; Singh et al., Biochemical Society Transactions Vol. 28 part 6 (2000), 925- 927; Liu et al.,  
20 al., Biochemical Society Transactions Vol. 28 part 6 (2000), 927-929; Smith et al., (Nature 407, (2000), 319-320; International Patent Application WO99/53050 A1). Sense and antisense sequences of the target gene(s) may also be expressed separately from one another by means of identical or different promoters (Nap, J-P et al, 6<sup>th</sup> International Congress of Plant  
25 Molecular Biology, Quebec, 18th-24th June, 2000; Poster S7-27, Session S7).

The reduction of the SSIII and/or the BEI and/or the BEII activity in the plant cells can thus also be achieved by generating double-stranded RNA  
30 molecules of SSIII and/or BEI and/or BEII genes. To this end, it is preferred to introduce, into the genome of plants, inverted repeats of DNA

molecules of SSIII and/or BEI and/or BEII genes or cDNAs, the DNA molecules to be transcribed (SSIII, BEI or BEII gene or cDNA, or fragments of these genes or cDNAs) being under the control of a promoter which governs the expression of said DNA molecules.

5

Moreover, it is known that the formation of double-stranded RNA molecules of promoter DNA molecules in plants *in trans* can lead to methylation and transcriptional inactivation of homologous copies of these promoters, hereinbelow referred to as target promoters (Mette et al.,

10 EMBO J. 19, (2000), 5194-5201).

Thus, it is possible, via the inactivation of the target promoter, to reduce the gene expression of a specific target gene (for example SSIII, BEI or BEII gene) which is naturally under the control of this target promoter.

This means that the DNA molecules which encompass the target  
15 promoters of the genes to be repressed (target genes) are in this case – in contrast to the original function of promoters in plants – not used as control elements for the expression of genes or cDNAs, but as transcribable DNA molecules themselves.

To generate the double-stranded target promoter RNA molecules *in*  
20 *planta*, where they may be present in the form of RNA hairpin molecules, it is preferred to use constructs which contain inverted repeats of the target promoter DNA molecules, the target promoter DNA molecules being under the control of a promoter which governs the gene expression of said target promoter DNA molecules. These constructs are subsequently introduced  
25 into the genome of plants. Expression of the inverted repeats of said target promoter DNA molecules leads to the formation of double-stranded target promoter RNA molecules *in planta* (Mette et al., EMBO J. 19, (2000), 5194-5201). The target promoter can thus be inactivated.

The reduction of the SSIII and/or BEI and/or BEII activity in the plant cells  
30 can thus also be achieved by generating double-stranded RNA molecules of promoter sequences of SSIII and/or BEI and/or BEII genes. To this end,

it is preferred to introduce, into the genome of plants, inverted repeats of promoter DNA molecules of SSIII and/or BEI and/or BEII promoters, the target promoter DNA molecules to be transcribed (SSIII, BEI and/or BEII promoter) being under the control of a promoter which governs the  
5 expression of said target promoter DNA molecules.

The skilled worker furthermore knows to achieve the activity of one or more SSIII, BEI and/or BEII proteins by expressing nonfunctional derivatives, in particular trans-dominant mutants, of such proteins and/or  
10 by expressing antagonists/inhibitors of such proteins.

Antagonists/inhibitors of such proteins encompass for example antibodies, antibody fragments or molecules with similar binding characteristics. For example, a cytoplasmic scFv antibody was employed for modulating the activity of the phytochrome A protein in genetically modified tobacco plants  
15 (Owen, *Bio/Technology* 10 (1992), 790-4; Review: Franken, E, Teuschel, U. and Hain, R., *Current Opinion in Biotechnology* 8, (1997), 411-416; Whitelam, *Trends Plant Sci.* 1 (1996), 268-272).

Useful promoters for the expression of nucleic acids which reduce the  
20 activity of a target gene are, for example, the promoter of the cauliflower mosaic virus 35S RNA and the maize ubiquitin promoter for constitutive expression, the patatin gene promoter B33 (Rocha-Sosa et al., *EMBO J.* 8 (1989), 23-29), the MCPI promoter of the potato metallopeptidase inhibitor gene (Hungarian Patent Application HU9801674) or the potato  
25 GBSSI promoter (International Patent Application WO 92/11376) for tuber-specific expression in potatoes, or a promoter which ensures expression uniquely in photosynthetically active tissues, for example the ST-LS1 promoter (Stockhaus et al., *Proc. Natl. Acad. Sci. USA* 84 (1987), 7943-7947; Stockhaus et al., *EMBO J.* 8 (1989), 2445-2451), the Ca/b promoter  
30 (see, for example, US 5656496, US 5639952, Bansal et al., *Proc. Natl. Acad. Sci. USA* 89, (1992), 3654-3658) and the Rubisco SSU promoter

(see, for example, US 5034322, US 4962028), or, for endosperm-specific expression, the glutelin promoter (Leisy et al., *Plant Mol. Biol.* 14, (1990), 41-50; Zheng et al., *Plant J.* 4, (1993), 357-366; Yoshihara et al., *FEBS Lett.* 383, (1996), 213-218), the Shrunk-1 promoter (Werr et al., *EMBO J.* 4, (1985), 1373-1380), the wheat HMG promoter, the USP promoter, the phaseolin promoter or promoters of zein genes from maize (Pedersen et al., *Cell* 29, (1982), 1015-1026; Quatroccio et al., *Plant Mol. Biol.* 15 (1990), 81-93).

The expression of the foreign nucleic acid molecule(s) is particularly advantageous in those plant organs which store starch. Examples of such organs are the tuber of the potato plant or the kernels, or endosperm, of maize, wheat or rice plants. This is why it is preferred to use promoters which confer expression in these organs.

However, it is also possible to use promoters which are activated only at a point in time determined by external factors (see, for example, WO 93/07279). Promoters which may be of particular interest in this context are promoters of heat-shock proteins, which permit simple induction. Others which can be [lacuna] are seed-specific promoters such as, for example, the *Vicia faba* USP promoter, which ensures seed-specific expression in *Vicia faba* and other plants (Fiedler et al., *Plant Mol. Biol.* 22, (1993), 669-679; Bäumlein et al., *Mol. Gen. Genet.* 225, (1991), 459-467). Fruit-specific promoters such as, for example, those described in WO91/01373 may furthermore also be employed.

Another element which may be present is a termination sequence, which serves for the correct termination of transcription and for the addition of a poly-A tail to the transcript, which is believed to have a function in stabilizing the transcripts. Such elements are described in the literature (cf., for example, Gielen et al., *EMBO J.* 8, (1989), 23-29) and can be substituted as desired.

The transgenic plant cells according to the invention synthesize a modified starch whose physico-chemical properties, in particular the amylose content and the amylose/amylopectin ratio, the phosphorus content, the viscosity behaviour, the gel strength, the granule size and/or the granule morphology is modified in comparison with starch synthesized in wild-type plants so that it is better suited to specific uses.

The present invention therefore also relates to a genetically modified plant cell according to the invention, in particular to a transgenic plant cell which synthesizes a modified starch.

Surprisingly, it has been found that the starch composition in the plant cells according to the invention is modified in such a way that the amylose content amounts to at least 30% and the phosphate content is increased and the end viscosity in the RVA analysis is increased in comparison with starch from plant cells from corresponding wild-type plants, so that this starch is better suited to specific uses.

In particular the starches according to the invention have the advantage that they gelatinize completely under standard conditions despite the increased amylose content. This markedly improves the processability of the starch in comparison with other starches with an increased amylose content. An increased temperature or increased pressure is therefore not necessary for gelatinizing the starch according to the invention. This is why the use of specific apparatuses such as, for example, jet cookers, extruders or autoclaves can be dispensed with when breaking down these starches. Another advantage of the starches according to the invention is that, when subjected to processing with hot rollers, they may be applied to the latter in the form of a suspension. Other starches with an increased amylose content would gelatinize, when subjected to this type of processing, to a limited extent only, if at all, and would not be capable of being applied to the rollers in question in the form of a paste or film.

The starches according to the invention are particularly suitable for all applications where the thickening ability, the gelling characteristics or the binding characteristics of added substances are of importance. The starch according to the invention is therefore particularly suitable for the production of foodstuffs such as, for example, baked goods, instant food, blancmange, soups, confectionery, chocolate, icecream, batter for fish or meat, frozen desserts or extruded snacks. Moreover, the starch according to the invention is suitable for the production of glues, for applications in textile processing, as additive to building materials, for applications in the field of animal nutrition, as additive for cosmetics, and in papermaking.

The starch which has been isolated from plant cells according to the invention is particularly suitable for the production of pregelatinized starch. Pregelatinized starches are physically modified starches which are produced predominantly by wet-heat treatment. As opposed to native starch, they form dispersions/pastes or gels with cold water, depending on the concentration of the pregelatinized starch used and as a function of the starch type used for producing the pregelatinized starch. Owing to these characteristics, a series of possible applications exist for pregelatinized starches in the food industry and in addition in many fields of industry. The use of pregelatinized starch, also referred to as cold-swelling starch, instead of native starch frequently has the advantage that production processes can be simplified and shortened.

The production of, for example, instant desserts and instant blancmange requires pregelatinized starches which, after being stirred into cold fluid such as, for example, water or milk, form firm gels in a short time as is the case with, for example, a blancmange which requires boiling. These demands are not met by the commercial pregelatinized starches made with wheat starch, potato starch or corn starch. To obtain the abovementioned characteristics, additives to the pregelatinized starch such as gelatin, alginate, carrageenan and/or inorganic salts are required in the case of the pregelatinized starches which are currently commercially

available. This addition of what are known as adjuvants is not required for example after the production of pregelatinized starches using starches according to the invention which are isolated from plant cells according to the invention.

5

The present invention also relates to a plant cell according to the invention with a modified starch with modified granule morphology.

For the purposes of the present invention, the term granule morphology is intended to refer to size and surface structure of native starch granules.

- 10 Starch is stored in the storage organs such as, for example, tubers, roots, embryos or endosperm of plants, as a crystalline structure in granular form. Starch granules in which these granular structures are retained after the starch has been isolated from plant cells are referred to as native starch. The mean granule size (determined by the method described
- 15 hereinbelow) of the native starch according to the invention is markedly lower than that of native starch isolated from wild-type plants. In the scanning electron micrograph (see Figs. 4 and 5) it can be seen clearly that, surprisingly, native starch granules according to the invention have a rough surface with many pores. The surface structure of native starch
- 20 granules isolated from wild-type plants, in contrast, is predominantly smooth in structure and no pores are discernible.

Both the presence of smaller granules and the rough surface with its pores lead to the fact that the surface area of starch granules according to the invention is considerably larger – at the same volume – than the surface

- 25 area of starch granules isolated from wild-type plants. The starch according to the invention is therefore particularly suited to the use as carrier for, for example, flavourings, pharmacologically active substances, prebiotics, probiotic microorganisms, enzymes or colorants. These starches are also particularly suitable for coagulating substances and in
- 30 papermaking.



A further possible application for the starches according to the invention is in the field of drilling for raw materials. Thus, when drilling for crude oil, adjuvants and/or lubricants must be employed which avoid overheating of the drill or drill column. Owing to its particular gelatinization properties, the  
5 starch according to the invention is therefore also particularly suited to the use in this field.

The present invention also relates to a plant cell according to the invention which contains a modified starch with an amylose content of at least 30%  
10 and which has an increased phosphate content and an increased final viscosity in the RVA analysis in comparison with starch from corresponding plant cells, from wild-type plants, which have not been genetically modified.

15 In connection with the present invention, the amylose content is determined by the method of Hovenkamp-Hermelink et al. (Potato Research 31, (1988), 241-246) described further below for potato starch. This method may also be applied to isolated starches from other plant species. Methods for isolating starches are known to the skilled worker.

20

For the purposes of the present invention, "phosphate content" of the starch refers to the content of phosphate covalently bonded in the form of starch phosphate monoesters.

In connection with the present invention, the term "increased phosphate  
25 content" means that the total phosphate content of covalently bonded phosphate and/or the phosphate content in C-6 position of the starch synthesized in the plant cells according to the invention is increased, by preference by at least 270%, more preferably by at least 300%, especially preferably by at least 350% in comparison with starch from plant cells of  
30 corresponding wild-type plants.

For the purposes of the present invention, the term "phosphate content in C6 position" is understood as meaning the content of phosphate groups which are bonded at the carbon atom position "6" of the glucose monomers of the starch. In principle, the positions C2, C3 and C6 of the  
5 glucose units can be phosphorylated in starch *in vivo*. In connection with the present invention, the determination of the phosphate content in C6 position (= C6-P content) can be carried out via the determination of glucose-6-phosphate by means of a visual-enzymatic test (Nielsen et al., Plant Physiol. 105, (1994), 111-117) (see below).

10

In connection with the present invention, the term "total phosphate content" of the starch refers to the content of phosphate bound covalently in C2, C3 and C6 position of the glucose units in the form of starch phosphate monoesters. The content of phosphorylated non-glucans such  
15 as, for example, phospholipids, does not come under the term "total phosphate content" in accordance with the invention. Phosphorylated non-glucans must therefore be removed quantitatively before determining the total phosphate content. Methods for separating the phosphorylated non-glucans (for example phospholipids) and the starch are known to the  
20 skilled worker. Methods for determining the total phosphate content are known to the skilled worker and described hereinbelow.

In a further embodiment of the invention, the plant cells according to the invention synthesize a starch which have a phosphate content of 40-  
25 120 nmol, in particular 60-110 nmol, preferably 80-100 C6-P per mg starch in C6 position of the glucose monomers of the starch.

A protocol for carrying out the RVA analysis is described further below. Mention must be made in particular that the RVA analysis of potato  
30 starches frequently operates with an 8% starch suspension (w/w). The documentation included with the apparatus "RVAsuper3" (instructions,

Newport Scientific Pty Ltd., Investment Support Group, Warriewood NSW 2102, Australia) recommends a suspension containing approximately 10% of starch for the analysis of potato starch.

Surprisingly, it has been found in the case of the starch from potato plants  
5 in relation to the present invention, that it was not possible to use an 8% starch suspension (2 g of starch in 25 ml of water) for the analysis since the final viscosity achieved values beyond the range of the apparatus. This is why only 6% starch suspensions (1.5 g of starch in 25 ml of water) were employed for the RVA analysis instead of 8% starch suspensions. In  
10 connection with the present invention, "increased end viscosity in the RVA analysis" is therefore understood as meaning an increase by at least 150%, especially by at least 200%, in particular by at least 250%, in comparison with wild-type plants which have not been genetically modified. The increase of the end viscosities relates to 6% starch  
15 suspensions in this context.

In connection with the present invention, a potato starch is furthermore understood as meaning one with an at least 300 RVU, especially 400 RVU, in particular 500 RVU final viscosity in the RVA analysis with a 6% starch content. The determination of the RVU values will be discussed  
20 in detail hereinbelow.

In a further preferred embodiment, the present invention relates to plant cells according to the invention which synthesize a modified starch which, after gelatinization in water, forms a gel with an increased gel strength in comparison with a gel made with starch of corresponding wild-type plant  
25 cells which have not been genetically modified.

For the purposes of the present invention, the term "increased gel strength" is understood as meaning an increase of the gel strength by preference by at least 300%, in particular by at least 500%, more  
30 preferably by at least 700% and especially preferably by at least 800%, up to a maximum of not more than 2000% or by not more than 1500% in

comparison with the gel strength of starch from corresponding wild-type plant cells which have not been genetically modified.

In connection with the present invention, the gel strength shall be determined with the aid of a Texture Analyser under the conditions

5 described hereinbelow.

To prepare starch gels, the crystalline structure of native starch must first be destroyed by heating in aqueous suspension with constant stirring. This was carried out with the aid of a Rapid Visco Analyser (Newport Scientific Pty Ltd., Investmet Support Group, Warriewood NSW 2102, Australia). As

10 already mentioned further above, the 8% starch suspension was replaced by an only 6% starch suspension in the case of starch from potato plants since the final viscosities of the 8% suspensions were outside the operating range of the apparatus. To determine gel strength, the starch suspensions gelatinized in the Rapid Visco Analyser were stored over a  
15 certain period and then subjected to analysis using a Texture Analyser. Accordingly, 8% gelatinized starch suspensions were also replaced by 6% gelatinized starch suspensions for determining gel strength.

In a further embodiment of the present invention, the modified starch  
20 synthesized in the plant cells according to the invention is distinguished not only by an increased amylose content in comparison with starch from corresponding wild-type plants and an increased phosphate content and an increased final viscosity in the RVA analysis, but also by a modified side chain distribution.

25

In a further embodiment, the present invention thus relates to plant cells according to the invention which synthesize a modified starch, the modified starch being characterized by a modified side chain distribution.

In one embodiment of the present invention, the term "modified side chain  
30 distribution" is understood as meaning a reduction of the amount of short side chains with a DP (= degree of polymerization) of 6 to 11 by at least

- 10%, preferably by at least 15%, in particular by at least 30% and especially preferably by at least 50% in comparison with the amount of short side chains with a DP of 6 to 11 of amylopectin from wild-type plants and/or an increase in the content of short side chains with a DP of 6 to 22
- 5 by at least 5%, preferably by at least 10%, in particular by at least 15% and especially preferably by at least 30% in comparison with the amount of short side chains with a DP of 16 to 22 of amylopectin from wild-type plants.
- 10 The amount of short side chains is determined via determining the percentage of a specific side chain in the total of all side chains. The total of all side chains is determined via determining the total area under the peaks which represent the degrees of polymerization of DP 6 to 26 in the HPLC chromatogram. The percentage of a particular side chain in the total
- 15 of all side chains is determined via determining the ratio of the area under the peak which represents this side chain in the HPLC chromatogram to the total area. A programme which may be used for determining the peak areas is, for example, Chromelion 6.20 from Dionex, USA.
- 20 In a further embodiment of the present invention, the modified starch synthesized in the plant cells according to the invention is distinguished not only by an increased amylose content in comparison with starch from corresponding wild-type plants and an increased phosphate content and an increased final viscosity in the RVA analysis, but also by a modified
- 25 "side chain profile DP 12 to 18" and/or by a modified "side chain profile DP 19 to 24" and/or by a modified "side chain profile DP 25 to 30" and/or by a modified "side chain profile DP 37 to 42" and/or by a modified "side chain profile DP 62 to 123".
- 30 In connection with the present invention, the term modified "side chain profile DP 12 to 18" is understood as meaning a reduction of the amount

of amylopectin side chains with a DP of 12 to 18 by at least 25%, preferably by at least 35%, especially preferably by at least 45% and very especially preferably by at least 55% in comparison with the amount of amylopectin side chains with a DP of 12 to 18 from wild-type plants.

5

In connection with the present invention, the term modified "side chain profile DP 19 to 24" is understood as meaning a reduction of the amount of amylopectin side chains with a DP of 19 to 24 by at least 10%, preferably by at least 20% and especially preferably by at least 30% in  
10 comparison with the amount of amylopectin side chains with a DP of 19 to 24 from wild-type plants.

In connection with the present invention, the term modified "side chain profile DP 25 to 30" is understood as meaning a reduction of the amount  
15 of amylopectin side chains with a DP of 25 to 30 by at least 5% in comparison with the amount of amylopectin side chains with a DP of 25 to 30 from wild-type plants.

In connection with the present invention, the term modified "side chain profile DP 37 to 42" is understood as meaning an increase of the amount  
20 of amylopectin side chains with a DP of 37 to 42 by at least 5%, preferably by at least 10% and especially preferably by at least 15% in comparison with the amount of amylopectin side chains with a DP of 37 to 42 from wild-type plants.

25

In connection with the present invention, the term modified "side chain profile DP 62 to 123" is understood as meaning an increase of the amount  
of amylopectin side chains with a DP of 62 to 123 by at least 20%, preferably by at least 35%, especially preferably by at least 50% in  
30 comparison with the amount of amylopectin side chains with a DP of 62 to 123 from wild-type plants.

The side chain profile is determined via determining the percentage of a specific group of side chains in the total of all side chains in the GPC chromatogram. To this end, the total area under the line of the GPC chromatogram is divided into individual segments, each of which  
5 represents groups of side chains of different lengths. The chosen segments contain side chains with the following degree of polymerization (DP = number of glucose monomers within one side chain): DP≤11, DP12-18, DP19-24, DP25-30, DP31-36, DP37-42, DP43-48, DP49-55, DP56-61 and DP62-123. To correlate the elution volume with the molecular mass,  
10 the GPC column used is calibrated with dextran standards (Fluka, Product# 31430). The dextrans used, their associated molecular mass and the elution volumes are shown in Fig. 9. Using the resulting calibration graph, the elution diagram is shown as a molecular weight distribution. To determine the molecular weight of the individual side chains, a molecular  
15 weight of 162 was set for glucose. The total area under the line in the GPC chromatogram is set as 100% and the percentage of the areas of the individual segments is calculated based on the percentage of the total area.

20 In a further especially preferred embodiment, the amylopectin of starch according to the invention, from plant cells according to the invention or plants according to the invention, shows an increased amount of the amylopectin side chains with a DP of greater than 123 in comparison with the amount of side chains with a DP of greater than 123 from amylopectin  
25 of wild-type plants.

The plant cells according to the invention may be used for the regeneration of intact plants.

The plants obtainable by regeneration of the transgenic plant cells according to the invention are likewise subject-matter of the present invention.

- 5 The plant cells according to the invention may belong to any plant species, i.e. to both monocotyledonous and dicotyledonous plants. They are preferably plant cells of agriculturally useful plants, i.e. plants which are grown by man for the purposes of nutrition or for technical, in particular industrial, purposes. The invention preferably relates to fibre-forming  
10 plants (for example flax, hemp, cotton), oil-storing plants (for example oilseed rape, sunflower, soybean), sugar-storing plants (for example sugar beet, sugar cane, sugar millet) and protein-storing plants (for example legumes).

In a further preferred embodiment, the invention relates to fodder plants, in particular to fodder grasses and forage grasses (alfalfa, clover and the  
15 like) and vegetable plants (for example tomato, lettuce, chicory).

In a further preferred embodiment, the invention relates to plant cells from starch-storing plants (for example wheat, barley, oats, rye, potato, maize, rice, pea, cassava), with plant cells from potato being especially preferred.

20

A multiplicity of techniques are available for introducing DNA into a plant host cell. These techniques encompass the transformation of plant cells with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as transformation agent, the fusion of protoplasts, injection, the  
25 electroporation of DNA, the introduction of the DNA by means of the biolistic approach, and other possibilities.

The use of the agrobacteria-mediated transformation of plant cells has been studied intensively and described sufficiently in EP 120516; Hoekema, IN: The Binary Plant Vector System Offsetdrukkerij Kanters  
30 B.V., Alblaserdam (1985), Chapter V; Fraley et al., Crit. Rev. Plant Sci. 4, 1-46 and in An et al. EMBO J. 4, (1985), 277-287. As regards the



transformation of potato, see, for example, Rocha-Sosa et al., EMBO J. 8, (1989), 29-33.).

The transformation of monocotyledonous plants by means of vectors  
5 based on transformation with agrobacterium has also been described  
(Chan et al., Plant Mol. Biol. 22, (1993), 491-506; Hiei et al., Plant J. 6,  
(1994) 271-282; Deng et al, Science in China 33, (1990), 28-34; Wilmink  
et al., Plant Cell Reports 11, (1992), 76-80; May et al., Bio/Technology 13,  
(1995), 486-492; Conner and Domisse, Int. J. Plant Sci. 153 (1992), 550-  
10 555; Ritchie et al, Transgenic Res. 2, (1993), 252-265). An alternative  
system for the transformation of monocotyledonous plants is the  
transformation by means of the biolistic approach (Wan and Lemaux, Plant  
Physiol. 104, (1994), 37-48; Vasil et al., Bio/Technology 11 (1993), 1553-  
1558; Ritala et al., Plant Mol. Biol. 24, (1994), 317-325; Spencer et al.,  
15 Theor. Appl. Genet. 79, (1990), 625-631), protoplast transformation, the  
electroporation of partially permeabilized cells, the introduction of DNA by  
means of glass fibres. In particular the transformation of maize has been  
described repeatedly in the literature (cf., for example, WO95/06128,  
EP0513849, EP0465875, EP0292435; Fromm et al., Biotechnology 8,  
20 (1990), 833-844; Gordon-Kamm et al., Plant Cell 2, (1990), 603-618;  
Koziel et al., Biotechnology 11 (1993), 194-200; Moroc et al., Theor. Appl.  
Genet. 80, (1990), 721-726).

The successful transformation of other cereal species has also been  
described, for example for barley (Wan and Lemaux, see above; Ritala et  
25 al., see above; Krens et al., Nature 296, (1982), 72-74) and wheat (Nehra  
et al., Plant J. 5, (1994), 285-297). All of the abovementioned methods are  
suitable for the purposes of the present invention.

Any promoter which is active in plant cells is generally suitable for the  
expression of the foreign nucleic acid molecule(s). The promoter may be  
30 chosen in such a way that expression in the plants according to the  
invention takes place constitutively or only in a specific tissue, at a

particular point in time of plant development or at a point in time determined by external factors. As regards the plant, the promoter may be homologous or heterologous.

- 5 In a further embodiment of the invention, at least one antisense RNA is expressed in plant cells in order to reduce the activity of one or more SSIII proteins and/or BEI proteins and/or BEII proteins.

The present invention therefore also relates to a plant cell according to the  
10 invention, wherein said foreign nucleic acid molecules are selected from the group consisting of

- 15 a) DNA molecules encoding at least one antisense RNA which brings about a reduction of the expression of at least one endogenous gene encoding SSIII proteins and/or BEI proteins and/or BEII proteins;
- b) DNA molecules which, via a cosuppression effect, lead to a reduction of the expression of at least one endogenous gene encoding SSIII protein(s) and/or BEI protein(s) and/or BEII protein(s);
- 20 c) DNA molecules encoding at least one ribozyme which specifically cleaves transcripts of at least one endogenous gene encoding SSIII proteins and/or BEI proteins and/or BEII proteins; and
- 25 d) Nucleic acid molecules introduced by means of in-vivo mutagenesis which lead to a mutation or insertion of a heterologous sequence in at least one endogenous gene encoding SSIII protein(s) and/or BEI protein(s) and/or BEII protein(s), the mutation or insertion bringing about a reduction of the expression  
30 of at least one gene encoding SSIII protein(s) and/or BEI

protein(s) and/or BEII protein(s), or the synthesis of inactive SSIII and/or BEI and/or BEII proteins;

- 5 e) DNA molecules which simultaneously encode at least one antisense RNA and at least one sense RNA, where said antisense RNA and said sense RNA form a double-stranded RNA molecule which brings about a reduction of the expression of at least one endogenous gene encoding SSIII protein(s) and/or BEI protein(s) and/or BEII protein(s);
- 10 f) DNA molecules containing transposons, the integration of the transposon sequences leading to a mutation or an insertion in at least one endogenous gene encoding SSIII protein(s) and/or BEI protein(s) and/or BEII protein(s) which brings about a reduction of the expression of at least one gene encoding an SSIII protein(s) and/or BEI protein(s) and/or BEII protein(s), or which results in the
- 15 synthesis of inactive SSIII and/or BEI and/or BEII proteins; and
- g) T-DNA molecules which, owing to the insertion into at least one endogenous gene encoding SSIII protein(s) and/or BEI protein(s) and/or BEII protein(s), brings about a reduction of the expression of at least one endogenous gene encoding SSIII protein(s) and/or
- 20 BEI protein(s) and/or BEII protein(s), or which result in the synthesis of inactive SSIII and/or BEI and/or BEII proteins.

In a further aspect, the present invention relates to any kind of propagation material of plants according to the invention.

25

A further aspect of the present invention relates to the use of the nucleic acid molecules described herein for the generation of the plant cells and plants according to the invention.

- 30 A further aspect of the present invention relates to a composition comprising at least one of the above nucleic acid molecules, where the at

least one nucleic acid molecule, after introduction into a plant cell, leads to the reduction of at least one SSIII protein which occurs endogenously in the plant cell and at least one BEII protein which occurs endogenously in the plant cell and preferably furthermore to the reduction of at least one  
5 BEI protein which occurs endogenously in the plant cell. The composition may comprise one or more nucleic acid constructs (cf. above).

A further aspect of the present invention relates to the use of the compositions according to the invention for the generation of the plant  
10 cells and plants according to the invention, and to a host cell, in particular a plant cell, containing the composition according to the invention.

Yet a further aspect of the present invention relates to a transformation system in plant cells, comprising at least one nucleic acid molecule and at  
15 least one plant cell, where the at least one nucleic acid molecule leads to the reduction of the activity of in each case at least one of the SSIII, BEI and BEII proteins which occur endogenously in the plant cell unless the activity of these proteins has been reduced already by an existing genetic modification of said plant cell. For the purposes of the present invention,  
20 "transformation system" thus relates to a combination of at least one plant cell to be transformed and at least one nucleic acid molecule as described above which is used for the transformation. Further components with which the skilled worker in the field of the transformation of plant cells is familiar and which are required in the transformation process, including  
25 buffers and the like, may be present in the transformation system according to the invention.

***Description of the figures*****Fig 1:**

A graphic representation of the viscosity characteristics of starch from potato plants. The analysis was carried out using a Rapid Visco Analyser (Newport Scientific Pty Ltd., Investmet Support Group, Warriewood NSW 2102, Australia). The conditions under which the analysis was carried out are described under RVA analytical method 1 in the chapter "General Methods". The test starch was isolated from tubers of wild-type plants (WT), plants with a reduced activity of an SSIII protein and of a BEI protein (038VL008 and 038VL107) or from plants with a reduced activity of an SSIII protein and a BEI protein and a BEII protein (110CF003 and 108CF041). The starch was isolated by the method described under "Examples", "Starch extraction process for potatoes".

15

**Fig 2:**

A graphic representation of the viscosity characteristics of starch from potato plants. The analysis was carried out using a Rapid Visco Analyser (Newport Scientific Pty Ltd., Investmet Support Group, Warriewood NSW 2102, Australia). The conditions under which the analysis was carried out are described under RVA analytical method 2 in the chapter "General Methods". The test starch was isolated from tubers of wild-type plants (WT), plants with a reduced activity of an SSIII protein and of a BEI protein (038VL008 and 038VL107) or from plants with a reduced activity of an SSIII protein and a BEI protein and a BEII protein (110CF003 and 108CF041). The starch was isolated by the method described under "Examples", "Starch extraction process for potatoes".

25

**Fig 3:**

A graphic representation of the viscosity characteristics of starch from potato plants. The analysis was carried out using a Rapid Visco Analyser

30

(Newport Scientific Pty Ltd., Investmet Support Group, Warriewod NSW 2102, Australia). The conditions under which the analysis was carried out are described under RVA analytical method 3 in the chapter "General Methods". The test starch was isolated from tubers of wild-type plants  
5 (WT), plants with a reduced activity of an SSIII protein and of a BEI protein (038VL008 and 038VL107) or from plants with a reduced activity of an SSIII protein and a BEI protein and a BEII protein (110CF003 and 108CF041). The starch was isolated by the method described under "Examples", "Starch extraction process for potatoes".

10

Fig 4:

Scanning-electron micrograph of a potato starch granule isolated from wild-type plants.

15 Fig 5:

Scanning-electron micrograph of a potato starch granule isolated from plants with a reduced activity of an SSIII protein and of a BEI protein and of a BEII protein (110CF003).

20 Fig 6:

Schematic representation of the vector pGSV71- $\alpha$ -BEII-basta, which was used for the retransformation of plants in which a reduced activity of an SSIII protein and of a BEI protein is already observed.

(RB, left T-DNA border, LB, right T-DNA border; CaMV35, cauliflower  
25 mosaic virus 35S promoter; NOS, polyadenylation sequence of the *Agrobacterium tumefaciens* nopaline synthase gene; OCS, polyadenylation sequence of the *Agrobacterium tumefaciens* octopine synthase gene; B33, promoter of the potato patatin gene; BEII, coding sequences of the potato BEII gene; bar, sequence encoding a  
30 *Streptomyces hygroscopicus* phosphinothricin acetyltransferase).

Fig 7:

Schematic representation of the vector pB33- $\alpha$ -BE- $\alpha$ -SSIII-Kan, which was used for the generation of transgenic plants with a reduced activity of an SSIII protein and a BEI protein (RB, left T-DNA border, LB, right T-DNA border; nos5', promoter of the *Agrobacterium tumefaciens* nopaline synthase gene; nptII, gene encoding the activity of a neomycin phosphotransferase; nos3, polyadenylation sequence of the *Agrobacterium tumefaciens* nopaline synthase gene; OCS, polyadenylation sequence of the *Agrobacterium tumefaciens* octopine synthase gene; B33, promoter of the potato patatin gene; BE, coding sequences of the potato BEI gene; SSIII, coding sequences of the potato SSIII gene).

Fig 8

The figure shows the entire elution diagram of the amylopectin from starches of lines 038VL008, 108CF041 and wild type. As shown in the figure, the amount of bigger side chains in line 108CF041 is markedly higher in contrast to the background 038VL008 and/or the corresponding wild type.

Fig 9

Calibration curve and table with corresponding dextran standards

Fig 10

The figure shows the entire elution diagram of the amylopectin from starches of lines 038VL008, 108CF041 and wild type. In contrast to Fig 8, the x axis does not show the elution volume, but the molecular weight. The elution diagram of Fig 8 as a function of the molecular weight distribution is shown with the aid of the calibration graph of Fig 9.

Fig 11

This represents the side chain profile distribution of the amylopectin from plants of line 038VL008 in comparison with the side chain profile of amylopectin from wild-type plants.

5 Fig 12

This represents the side chain profile distribution of the amylopectin from plants of line 108CF041 in comparison with the side chain profile of amylopectin from wild-type plants.

10 **Description of the sequences**

Seq ID 1:

Nucleic acid sequence of the potato (*Solanum tuberosum*) starch synthase SSIII with indication of the sequences which encode the corresponding

15 SSIII protein.

Seq ID 2:

Amino acid sequence of a potato SSIII protein.

Seq ID 3:

Amino acid sequence of the Pfam cbm25 binding domain of the potato

20 SSIII protein (*Solanum tuberosum*).

Seq ID 4:

Coding nucleic acid sequence of the potato (*Solanum tuberosum*) branching enzyme BEI.

Seq ID 5:

25 Amino acid sequence of the potato (*Solanum tuberosum*) branching enzyme BEI.

Seq ID 6:

Coding nucleic acid sequence of the potato (*Solanum tuberosum*) branching enzyme BEII.

30 Seq ID 7:



Amino acid sequence of the potato (*Solanum tuberosum*) branching enzyme BEII.

Seq ID 8:

PCR-amplified nucleic acid sequence of the potato (*Solanum tuberosum*)

5 branching enzyme BEII.

### **General Methods**

The following methods were used in the examples:

5

#### Starch analysis

##### a) Determination of the amylose content and of the amylose/amylopectin ratio

10 Starch was isolated from potato plants by standard methods, and the amylose content and the amylose:amylopectin ratio was determined by the method described by Hovenkamp-Hermelink et al. (Potato Research 31, (1988), 241-246).

##### b) Determination of the phosphate content

15 In starch, the positions C2, C3 and C6 of the glucose units can be phosphorylated. To determine the C6-P content of starch, 50 mg of starch are hydrolysed for 4 h at 95°C in 500 µl of 0.7 M HCl. The samples are then centrifuged for 10 minutes at 15 500 g and the supernatants are removed. 7 µl of the supernatants are mixed with 193 µl of imidazole  
20 buffer (100 mM imidazole, pH 7.4; 5 mM MgCl<sub>2</sub>, 1 mM EDTA and 0.4 mM NAD). The measurement was carried out in a photometer at 340 nm. After the base absorption had been established, the enzyme reaction was started by addition of 2 units glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*, Boehringer Mannheim). The change in  
25 absorption is directly proportional to the concentration of the G-6-P content of the starch.

The total phosphate content was determined by the method of Ames (Methods in Enzymology VIII, (1966), 115-118).

30 Approximately 50 mg of starch are treated with 30 µl of ethanolic magnesium nitrate solution and ashed for 3 hours at 500°C in a muffle

oven. The residue is treated with 300 µl of 0.5 M hydrochloric acid and incubated for 30 minutes at 60°C. One aliquot is subsequently made up to 300 µl 0.5 M hydrochloric acid and this is added to a mixture of 100 µl of 10% ascorbic acid and 600 µl of 0.42% ammonium molybdate in 2 M sulphuric acid and incubated for 20 minutes at 45°C.

This is followed by a photometric determination at 820 nm with a phosphate calibration series as standard.

c) Determination of the gel strength (Texture Analyser)

1.5 g of starch (DM) are gelatinized in the RVA apparatus in 25 ml of an aqueous suspension (temperature programme: see item d) "Determination of the viscosity characteristics by means of a Rapid Visco Analyser (RVA)") and subsequently stored for 24 hours at room temperature in a sealed container. The samples are fixed under the probe (round piston with planar surface) of a Texture Analyser TA-XT2 from Stable Micro Systems (Surrey, UK) and the gel strength was determined using the following parameters:

-	Test speed	0.5 mm/s
-	Depth of penetration	7 mm
-	Contact surface	113 mm <sup>2</sup>
-	Pressure	2 g

d) Determination of the viscosity characteristics by means of a Rapid Visco Analyser (RVA)

Standard method

2 g of starch (DM) are taken up in 25 ml of H<sub>2</sub>O (VE-type water, conductivity of at least 15 mega ohm) and used for the analysis in a Rapid Visco Analyser (Newport Scientific Pty Ltd., Investmet Support Group, Warriewood NSW 2102, Australia). The apparatus is operated following the manufacturer's instructions. The viscosity values are indicated in RVUs in

accordance with the manufacturer's operating manual, which is incorporated into the description herewith by reference. To determine the viscosity of the aqueous starch solution, the starch suspension is first heated for one minute at 50°C (step 1), and then heated from 50°C to 5 95°C at a rate of 12°C per minute (step 2). The temperature is then held for 2.5 minutes at 95°C (step 3). Then, the solution is cooled from 95°C to 50°C at a rate of 12°C per minute (step 4). The viscosity is determined during the entire duration.

10 In particular in those cases where the limits of the measuring range of the RVA were insufficient when 2.0 g (DM) of starch in 25 ml of H<sub>2</sub>O (VE-type water, conductivity of at least 15 mega ohm) were weighed in, only 1.5 g of starch (DM) were taken up in 25 ml of H<sub>2</sub>O (VE-type water, conductivity of at least 15 mega ohm).

15

For reasons of comparison with the prior art, a modified temperature profile was additionally used in some cases.

The following temperature profiles were used:

20 RVA analytical method 1:

To determine the viscosity of a 6% aqueous starch solution, the starch suspension is first stirred for 10 seconds at 960 rpm and subsequently heated at 50°C at a stirring speed of 160 rpm, initially for a minute (step 1). The temperature was then raised from 50°C to 95°C at a heating rate of 25 12°C per minute (step 2). The temperature is held for 2.5 minutes at 95°C (step 3) and then cooled from 95°C to 50°C at 12°C per minute (step 4). In the last step (step 5), the temperature of 50°C is held for 2 minutes.

After the programme has ended, the stirrer is removed and the beaker covered. The gelatinized starch is now available for the texture analysis 30 after 24 hours.

#### RVA analytical method 2:

- To determine the viscosity of a 6% aqueous starch solution, the starch suspension is first stirred for 10 seconds at 960 rpm and subsequently heated at 50°C at a stirring speed of 160 rpm, initially for two minutes
- 5 (step 1). The temperature was then raised from 50°C to 95°C at a heating rate of 1.5°C per minute (step 2). The temperature is held for 15 minutes at 95°C (step 3) and then cooled from 95°C to 50°C at 1.5°C per minute (step 4). In the last step (step 5), the temperature of 50°C is held for 15 minutes.
- 10 After the programme has ended, the stirrer is removed and the beaker covered. The gelatinized starch is now available for the texture analysis after 24 hours.

#### RVA analytical method 3:

- 15 To determine the viscosity of a 10% aqueous starch solution, the starch suspension is first stirred for 10 seconds at 960 rpm and subsequently heated at 50°C at a stirring speed of 160 rpm, initially for two minutes (step 1). The temperature was then raised from 50°C to 95°C at a heating rate of 1.5°C per minute (step 2). The temperature is held for 15 minutes
- 20 at 95°C (step 3) and then cooled from 95°C to 50°C at 1.5°C per minute (step 4). In the last step (step 5), the temperature of 50°C is held for 15 minutes. This profile of the RVA analysis corresponds to the one employed in WO 9634968.
- After the programme has ended, the stirrer is removed and the beaker
- 25 covered. The gelatinized starch is now available for the texture analysis after 24 hours.

- The profile of the RVA analysis contains parameters which are shown for the comparison of different measurements and substances. In the context
- 30 of the present invention, the following terms are to be understood as follows:

1. Maximum viscosity (RVA Max)

The maximum viscosity is understood as meaning the highest viscosity value, measured in RVUs, obtained in step 2 or 3 of the temperature profile.

5 2. Minimum viscosity (RVA Min)

The minimum viscosity is understood as meaning the lowest viscosity value, measured in RVUs, observed in the temperature profile after the maximum viscosity. Normally, this takes place in step 3 of the temperature profile.

10 3. Final viscosity (RVA Fin)

The final viscosity is understood as meaning the viscosity value, measured in RVUs, observed at the end of the measurement.

4. Setback (RVA Set)

15 What is known as the "setback" is calculated by subtracting the value of the final viscosity from that of the minimum occurring after the maximum viscosity in the curve.

5. Gelatinization temperature (RVA T)

20 The gelatinization temperature is understood as meaning the point in time of the temperature profile where, for the first time, the viscosity increases drastically for a brief period.

e) Analysis of the side-chain distribution of the amylopectin by means of ion-exchange chromatography

25 To separate amylose and amylopectin, 200 mg of starch are dissolved in 50 ml reaction vessels, using 12 ml of 90% (v/v) DMSO in H<sub>2</sub>O. After addition of 3 volumes of ethanol, the precipitate is separated by centrifugation for 10 minutes at about 1800 g at room temperature (RT). The pellet is then washed with 30 ml of ethanol, dried and  
30 dissolved in 40 ml of 1% (w/v) NaCl solution at 75°C. After the solution has cooled to 30°C, approximately 90 mg of thymol are added slowly,

and this solution is incubated for at least 60 h at 30°C. The solution is then centrifuged for 30 minutes at 2000 g (RT). The supernatant is then treated with 3 volumes of ethanol, and the amylopectin which settles out is separated by centrifugation for 5 minutes at 2000 g (RT).

- 5 The pellet (amylopectin) is then washed with ethanol and dried using acetone. By addition of DMSO to the pellet, one obtains a 1% solution, of which 200 µl are treated with 345 µl of water, 10 µl of 0.5 M sodium acetate (pH 3.5) and 5 µl of isoamylase (dilution 1:10; Megazyme) and incubated for about 16 hours at 37°C. A 1:5 aqueous dilution of this
- 10 digest is subsequently filtered through a 0.2 µm filter, and 100 µl of the filtrate are analysed by ion chromatography (HPAEC-PAD, Dionex). Separation was performed using a PA-100 column (with suitable precolumn), while detection was performed amperometrically. The elution conditions were as follows:

15 Solution A - 0.15M NaOH

Solution B – 1 M sodium acetate in 0.15M NaOH

t (min)	Solution A (%)	Solution B (%)
5	0	100
35	30	70
45	32	68
60	100	0
70	100	0
72	0	100
80	0	100
Stop		

**Table 1:** Composition of the elution buffer for the side chain analysis of the amylopectin at different times during the HPEAC-PAD Dionex analysis. Between the times stated, the composition of the elution buffer changes in each case linearly.

The determination of the relative amount of short side chains in the total of all side chains is carried out via the determination of the percentage of a particular side chain in the total of all side chains. The total of all side chains is determined via the determination of the total area under the peaks which represent the polymerization degrees of DP6 to 26 in the HPCL chromatogram.

The percentage of a particular side chain in the total of all side chains is determined via the determination of the ratio of the area under the peak which represents this side chain in the HPLC chromatogram to the total area. The programme Chromelion 6.20 Version 6.20 from Dionex, USA, was used for determining the peak areas.

f) Granule size determination

Starch was extracted from potato tubers by standard methods (see Examples).

The granule size determination was then carried out using a photosedimentometer of type "Lumosed FS1" from Retsch GmbH, Germany, using the software V.2.3. The software settings were as follows:

20	Substance data:	Calibration No. 0	
		Density [kg/m <sup>3</sup> ] 1500	
	Sedimentation fluid:	Type Water	
		Viscosity [Pa s]	0.001
		Density [kg/m <sup>3</sup> ]	1000
25		Addition	-
		Recordings	5 min
		Cut-off [µm]	250
		Passage [%]	100
		Measuring range	4.34 -
30	117.39 µm	Calibration	N



Temperature 20°C

- 5 The granule size distribution was determined in aqueous solution and was carried out following the manufacturer's instructions and on the basis of the literature by, for example, H. Pitsch, Korngrößenbestimmung [granule size determination]; LABO-1988/3 Fachzeitschrift für Labortechnik, Darmstadt.

g) Scanning electron micrographs (SEM)

- 10 To study the surface of the starch samples, the latter were dusted onto the sample holder using a conductive adhesive. To avoid charging, the sample holders were finally sputtered with a 4 nm Pt coating. The starch samples were studied using the field emission scanning electron microscope JSM 6330 F (Jeol) at an accelerating voltage of 5 kV.

- 15 h) Determination of the activity of the SSIII, BEI and BEII proteins  
These were carried out as specified in the examples.

## Examples

### Generation of the expression vector ME5/6

- pGSV71 is a derivative of the plasmid pGSV7, which is derived from the
- 5 intermediary vector pGSV1. pGSV1 is a derivative of pGSC1700, whose construction has been described by Cornelissen and Vanderwiele (Nucleic Acid Research 17, (1989), 19-25). pGSV1 was obtained from pGSC1700 by deleting the carbenicillin resistance gene and deleting the T-DNA sequences of the TL-DNA region of the plasmid pTiB6S3.
- 10 pGSV7 contains the replication origin of the plasmid pBR322 (Bolivar et al., Gene 2, (1977), 95-113) and the replication origin of the *Pseudomonas* plasmid pVS1 (Itoh et al., Plasmid 11, (1984), 206). pGSV7 additionally contains the selectable marker gene *aadA*, from the *Klebsiella pneumoniae* transposon Tn1331, which confers resistance to the
- 15 antibiotics spectinomycin and streptomycin (Tolmasky, Plasmid 24 (3), (1990), 218-226; Tolmasky and Crosa, Plasmid 29(1), (1993), 31-40)
- The plasmid pGSV71 was obtained by cloning a chimeric *bar* gene between the border regions of pGSV7. The chimeric *bar* gene contains the cauliflower mosaic virus promoter sequence for transcriptional initiation
- 20 (Odell et al., Nature 313, (1985), 180), the *Streptomyces hygroscopicus* *bar* gene (Thompson et al., Embo J. 6, (1987), 2519-2523) and the 3'-untranslated region of the pTiT37 T-DNA nopal synthase gene for transcriptional termination and for polyadenylation. The *bar* gene confers tolerance to the herbicide glufosinate-ammonium.
- 25 The T-DNA contains the right border sequence of the TL-DNA from the plasmid pTiB6S3 (Gielen et al., EMBO J. 3, (1984), 835-846) at position 198-222. A polylinker sequence is located between nucleotide 223-249. The nucleotides 250-1634 contain the cauliflower mosaic virus p35S3 promoter region (Odell et al., see above). The coding sequence of the
- 30 *Streptomyces hygroscopicus* phosphinothricin resistance gene (*bar*) (Thompson et al. 1987, see above) is arranged between the nucleotides

- 1635-2186. The two terminal codons at the 5' end of the *bar* wild-type gene were replaced by the codons ATG and GAC. A polylinker sequence is located between the nucleotides 2187-2205. The 260 bp *TaqI* fragment of the untranslated 3' end of the nopaline synthase gene (3'nos) from the T-DNA of the plasmid pTiT37 (Depicker et al., J. Mol. Appl. Genet. 1, (1982), 561-573) is located between the nucleotides 2206 and 2465. The nucleotides 2466-2519 contain a polylinker sequence. The left border sequence of the pTiB6S3 TL-DNA (Gielen et al., EMBO J. 3, (1984), 835-846) is located between the nucleotides 2520-2544.
- 10 The vector pGSV71 was then cut using the enzyme *PstI* and made blunt-ended. The B33 promoter and the *ocs* cassette was then excised from the vector pB33-Kan in the form of an *EcoRI-HindIII* fragment, made blunt-ended and inserted into the vector pGSV71 which had been cut with *PstI* and made blunt-ended. The resulting vector was used as starting vector
- 15 for the construction of ME5/6: An oligonucleotide containing the cleavage sites *EcoRI*, *PacI*, *SpeI*, *SrfI*, *SpeI*, *NofI*, *PacI* and *EcoRI* was introduced into the *PstI* cleavage site of the vector ME4/6 located between the B33 promoter and the *ocs* element, duplicating the *PstI* cleavage site. The resulting expression vector was termed ME5/6.

20

#### Description of the vector pSK-Pac:

pSK-Pac is a derivative of pSK-Bluescript (Stratagene, USA) in which a *PacI* cleavage site was introduced at each flank of the multiple cloning site (MCS).

25

#### **Generation of transgenic potato plants with a reduced gene expression of a BEI, SSIII and BEII gene**

- 30 To generate transgenic plants with a reduced activity of a BEI, an SSIII and a BEII protein, transgenic plants with a reduced activity of a BEI and

an SSIII protein were generated in a first step. To this end, the T-DNA of the plasmid pB33- $\alpha$ BEI- $\alpha$ SSIII-Kan was transferred into potato plants with the aid of agrobacteria as described by Rocha-Sosa et al. (EMBO J. 8, (1989), 23-29).

- 5 To construct the plasmid pB33- $\alpha$ BEI- $\alpha$ SSIII-Kan (see Fig 7), the expression vector pBin33-Kan was constructed in a first step. To this end, the promoter of the *Solanum tuberosum* patatin gene B33 (Rocha-Sosa et al., 1989, see above) was ligated in the form of a *DraI* fragment (nucleotides -1512 - +14) into the *SstI*-cut vector pUC19 (Genbank Acc. No. M77789), whose ends have been made blunt-ended with the aid of T4 DNA polymerase. This gave rise to the plasmid pUC19-B33. The B33 promoter was excised from this plasmid using *EcoRI* and *SmaI* and ligated into the suitably cut vector pBinAR. This gave rise to the plant expression vector pBin33-Kan. The plasmid pBinAR is a derivative of the vector
- 10 plasmid pBin19 (Bevan, Nucl. Acid Research 12, (1984), 8711-8721) and was constructed by Höfgen and Willmitzer (Plant Sci. 66, (1990), 221-230). A 1631 bp *HindIII* fragment which contains a partial cDNA encoding the potato BEI enzyme (Kossmann et al., 1991, Mol. & Gen. Genetics 230(1-2):39-44) was then made blunt-ended and introduced into the vector
- 15 pBin33, which had previously been cut with *SmaI*, in antisense orientation with regard to the B33 promoter (promoter of the *Solanum tuberosum* patatin gene B33; Rocha-Sosa et al., 1989). The resulting plasmid was cut open using *BamHI*. A 1363 bp *BamHI* fragment containing a partial cDNA encoding the potato SSIII enzyme (Abel et al., 1996, loc.cit.) was
- 20 introduced into the cleavage site, again in antisense orientation with regard to the B33 promoter.
- 25

After the transformation, various lines of transgenic potato plants in whose tubers a markedly reduced activity of a BEI and SSIII protein was

30 observed were identified. The plants resulting from this transformation were termed 038VL.

To detect the activity of soluble starch synthases (SSIII) by non-denaturing gel electrophoresis, tissue samples of potato tubers were digested in 50 mM Tris-HCl pH 7.6, 2 mM DTT, 2.5 mM EDTA, 10% glycerol and 0.4 mM PMSF. The electrophoresis was carried out in a MiniProtein II chamber  
5 (BioRAD). The monomer concentration of the gels, which had a thickness of 1.5 mm, amounted to 7.5% (w/v), and 25 mM Tris-Glycin pH 8.4 acted as the gel and the running buffers. Identical amounts of protein extract were applied and separated for 2 h at 10 mA for each gel.

The activity gels were subsequently incubated in 50 mM Tricine-NaOH pH  
10 8.5, 25 mM potassium acetate, 2 mM EDTA, 2 mM DTT, 1 mM ADP-glucose, 0.1% (w/v) amylopectin and 0.5 M sodium citrate. Glucans formed were stained with Lugol's solution.

The BEI activity was likewise detected with the aid of non-denaturing gel electrophoresis:

15 To isolate proteins from plants, the sample material was comminuted in liquid nitrogen using a pestle and mortar, taken up in extraction buffer (50 mM sodium citrate, pH 6.5; 1 mM EDTA, 4 mM DTT), centrifuged (10 min, 14,000 g, 4°C) and then employed directly in the protein concentration measurement following the method of Bradford. Then, 5 to 20 µg of total  
20 protein extract (as required) were treated with 4x loading buffer (20% glycerol, 125 mM Tris HCl, pH 6.8) and applied to a BE activity gel. The running buffer (RB) was composed as follows: RB = 30.2 g Tris-base, pH 8.0, 144 g glycine per 1 l H<sub>2</sub>O.

After running of the gel had ended, each of the gels was incubated  
25 overnight at 37°C in 25 ml of "phosphorylase buffer" (25 ml 1M sodium citrate pH 7.0, 0.47 g glucose-1-phosphate, 12.5 mg AMP, 2.5 mg phosphorylase a/b from rabbit). The gels were stained using Lugol's solution.

30 More in-depth analyses demonstrated that isolated starches from lines 038VL008 and 038VL107, in which both the BEI and the SSIII protein

were reduced, showed the highest phosphate content of all independent transformants studied.

Plants of these lines were subsequently transformed with the plasmid pGSV71- $\alpha$ BEII-basta as described by Rocha-Sosa et al. (EMBO J. 8, 5 (1989), 23-29).

Plasmid pGSV71- $\alpha$ BEII-basta was constructed by screening a tuber-specific potato cDNA library with a DNA fragment amplified using RT-PCR (Primer: 5'-gggggtgttggttgacta and 5'-cccttcctcctaatccca; Stratagene ProSTAR™ HF Single-Tube RT-PCR system) with total RNA from tubers as template, following standard methods. In this manner, an approximately 1250 bp DNA fragment (SEQ ID No. 8) was isolated and then subcloned into the EcoRV cleavage site of the cloning vector pSK-Pac (see hereinabove) in the form of an EcoRV-SmaI fragment and subsequently ligated into the expression vector ME5/6 in antisense orientation relative to the promoter in the form of a PacI fragment. This gave rise to the plasmid pGSV71- $\alpha$ BEII-basta (see Fig 6).

Tuber tissue samples of the independent transformants were obtained from the plants obtained by transformation with the plasmid pGSV71- $\alpha$ BEII-basta, which were referred to as 108CF and 110CF, and their amylose content was determined (see Methods). The starches from the independent lines whose tubers had the highest amylose content were used for a further analysis of the starch characteristics. To prove that in these plants not only the activity of a BEI and SSIII protein is reduced, but also that the activity of a BEII protein is reduced, another analysis was carried out with the aid of non-denaturing gel electrophoresis. The analysis was carried out following the same method as already carried out above for the analysis of the reducing BEI activity, with the exception that the non-denaturing polyacrylamide gel contained 0.5% of maltodextrin (Beba, 15% strength maltodextrin solution for newborns, Nestle) in addition to the above-described composition. The dextrin addition made it possible to

show the different activities of the BEI and BEII proteins after incubation of the gels in "phosphorylase buffer" (25 ml 1M sodium citrate pH 7.0, 0.47 g glucose-1-phosphate, 12.5 mg AMP, 2.5 mg phosphorylase a/b from rabbit) overnight at 37°C, followed by staining with Lugol's solution in a gel.

### ***Potato starch extraction process***

All tubers of one line (4 to 5 kg) are processed jointly in a commercially available juice extractor (Multipress automatic MP80, Braun). The starch-containing fruit water is collected in a 10-l bucket (ratio bucket height: bucket diameter = approx. 1.1) containing 200 ml of tap water together with a spoon-tipful (approx. 3-4 g) of sodium disulphite. The bucket is subsequently filled completely with tap water. After the starch has been allowed to settle for 2 hours, the supernatant is decanted off, the starch is resuspended in 10 l of tap water and poured over a sieve with a mesh size of 125 µm. After 2 hours (starch has again settled at the bottom of the bucket), the aqueous supernatant is again decanted off. This wash step is repeated 3 more times so that the starch is resuspended a total of 5 times in fresh tap water. Thereafter, the starches are dried at 37°C to a water content of 12-17% and homogenized using a pestle and mortar. The starches are now available for analyses.

### **Example 2**

25

#### **Analysis of the starch from plants with reduced BEI, SSIII and BEII gene expression**

The starch from various independent lines of the transformations 108CF and 110CF described in Example 1 were isolated from potato tubers. The physico-chemical properties of this starch were subsequently analysed. The results of the characterization of the modified starches are shown in

Table 2 (Tab. 2) for an example of a selection of certain plant lines. The analyses were carried out by the methods described hereinabove.



Tables 2, 3 and 4 which follow summarize the results of the RVA analysis based on starch from wild-type plants:

**RVA analytical method 1**

	RVA Max (%)	RVA Min (%)	RVA Fin (%)	RVA Set (%)	RVA T (%)	Gel strength
cv.Desiree	100	100	100	100	100	100
038VL008	158.7	69.8	72.0	79.5	73.0	55.4
108CF041	59.6	89.9	227.5	693.7	150.2	532.3
038VL107	151.1	94.3	94.0	93.0	82.2	52.2
110CF003	106.4	158.6	265.0	625.7	151.5	737.1

**Table 2:** Parameters of the RVA analysis of starch isolated from wild-type plants (cv.

- 5 Desiree), plants with a reduced activity of an SSIII and a BEI protein (038VL008, 038VL107), and of plants with a reduced activity of an SSIII and of a BEI and of a BEII protein (108CF041, 110CF003) in per cent based on data of starch of the wild type. The RVA analysis was carried out as described in Analytical method 1.

**10 RVA analytical method 2**

	RVA Max (%)	RVA Min (%)	RVA Fin (%)	RVA Set (%)	RVA T (%)	Gel strength
cv. Desiree	100	100	100	100	100	100
038VL008	167.1	40.4	52.6	77.6	54.2	63.0
108CF041	44.5	82.5	187.5	402.7	137.4	412.2
038VL107	152.0	76.1	81.9	93.8	76.9	51.7
110CF003	92.4	172.2	n.d.	n.d.	139.0	795.0

**Table 3:** Parameters of the RVA analysis of starch isolated from wild-type plants (cv.

- Desiree), plants with a reduced activity of an SSIII and a BEI protein (038VL008, 038VL107), and of plants with a reduced activity of an SSIII and of a BEI and of a BEII protein (108CF041, 110CF003) in per cent based on data of starch of the wild type. The
- 15 RVA analysis was carried out as described in Analytical method 2.

**RVA analytical method 3**

	RVA Max (%)	RVA Min (%)	RVA Fin (%)	RVA Set (%)	RVA T (%)	Gel strength
cv. Desiree	100	100	100	100	100	100
038VL008	n.d.	50.2	76.5	127.8	77.0	100.5

108CF041	74.7	291.0	n.d.	205.7	236.0	630.3
038VL107	n.d.	84.5	86.4	90.1	102.3	58.1
110CF003	89.8	259.7	n.d.	n.d.	196.6	663.9

**Table 4:** Parameters of the RVA analysis of starch isolated from wild-type plants (cv. Desiree), plants with a reduced activity of an SSIII and a BEI protein (038VL008, 038VL107), and of plants with a reduced activity of an SSIII and of a BEI and of a BEII protein (108CF041, 110CF003) in per cent based on data of starch of the wild type. The

5 RVA analysis was carried out as described in Analytical method 3.

The following tables 5, 6 and 7 summarize the results of the RVA analysis.

The data do not refer to the wild type, but are the actual measurements:

#### 10 RVA analytical method 1 (see also Fig. 1)

	RVA Max (RVU)	RVA Min (RVU)	RVA Fin (RVU)	RVA Set (RVU)	RVA T (RVU)	Gel strength
cv. Desiree	255.05	162.33	210.25	47.92	4.6	25.1
038VL008	404.83	113.25	151.33	38.08	3.36	13.9
108CF041	152.08	145.92	478.33	332.42	6.91	133.6
038VL107	385.5	153	197.58	44.58	3.78	13.1
110CF003	271.5	257.42	557.25	299.83	6.97	185

**Table 5:** Parameters of the RVA analysis of starch isolated from wild-type plants (cv. Desiree), plants with a reduced activity of an SSIII and a BEI protein (038VL008, 038VL107), and of plants with a reduced activity of an SSIII and of a BEI and of a BEII protein (108CF041, 110CF003) in RVUs. The RVA analysis was carried out as described

15 in Analytical method 1.

#### RVA analytical method 2 (see also Fig. 2)

	RVA Max (RVU)	RVA Min (RVU)	RVA Fin (RVU)	RVA Set (RVU)	RVA T (RVU)	Gel strength
cv. Desiree	212.17	113.75	169.25	55.5	28.78	23.8
038VL008	354.58	45.92	89	43.08	15.61	15
108CF041	94.33	93.83	317.33	223.5	39.53	98.1
038VL107	322.58	86.58	138.67	52.08	22.13	12.3
110CF003	196.08	195.92	n.d.	n.d.	39.99	189.2

**Table 6:** Parameters of the RVA analysis of starch isolated from wild-type plants (cv. Desiree), plants with a reduced activity of an SSIII and a BEI protein (038VL008, 038VL107), and of plants with a reduced activity of an SSIII and of a BEI and of a BEII protein (108CF041, 110CF003) in RVUs. The RVA analysis was carried out as described in Analytical method 2.

5

RVA analytical method 3 (see also Fig. 3)

	RVA Max (RVU)	RVA Min (RVU)	RVA Fin (RVU)	RVA Set (RVU)	RVA T (RVU)	Gel strength
Desiree	819.67	207.67	314.25	106.58	16.88	56.5
038VL008	n.d.	104.17	240.33	136.17	12.99	56.8
108CF041	612.33	604.25	823.5	219.25	39.83	356.1
038VL107	n.d.	175.42	271.5	96.08	17.27	32.8
110CF003	736.08	539.42	n.d.	n.d.	33.18	375.1

**Table 7:** Parameters of the RVA analysis of starch isolated from wild-type plants (cv. Desiree), plants with a reduced activity of an SSIII and a BEI protein (038VL008, 038VL107), and of plants with a reduced activity of an SSIII and of a BEI and of a BEII protein (108CF041, 110CF003) in RVUs. The RVA analysis was carried out as described in Analytical method 3.

10

Summary of the phosphate and amylose analyses:

No.	Genotype	Phosphate in C6 (%)	Total phosphate in (%)	Amylose (%)	Amylose (% WT)
1	cv. Desiree	100	100	22	100
2	038VL008	346.4	255.2	19.4	85.8
3	108CF041	557.3	427.6	36.8	162.8
4	038VL107	225.5	182.8	19.7	87.2
5	110CF003	446.4	348.3	34.6	153.1

**Table 8:** Phosphate and amylose contents of starch isolated from wild-type plants (cv. Desiree), plants with a reduced activity of an SSIII and a BEI protein (038VL008, 038VL107) and of plants with a reduced activity of an SSIII and of a BEI and of a BEII protein (108CF041, 110CF003). The phosphate contents in the C6 position of the

15

glucose monomers and the total phosphate content of the starch are indicated in per cent based on starch from wild-type plants; amylose contents are indicated in per cent amylose based on the total amount of the starch, or in per cent based on the amylose content of starch from wild-type plants.

5

The analysis of the side-chain distribution of the amylopectin was carried out as described above. The table which follows is a summary of the contributions of the individual peak areas:

10

Glucose units	cv. Desiree	038VL 008	108CF 041	038VL 107	110CF 003
dp 6	1.52	4.16	1.88	2.39	0.86
dp 7	1.4	1.4	0.63	1.42	0.59
dp 8	1.23	0.77	0.33	0.99	0.38
dp 9	2.05	1.42	0.74	1.79	0.75
dp 10	3.55	2.8	1.74	3.33	1.77
dp 11	5.16	4.41	2.92	4.96	3.46
dp 12	6.25	5.77	4.47	6.22	5.17
dp 13	6.71	6.7	5.63	6.87	6.35
dp 14	6.75	7.06	6.35	6.99	7.38
dp 15	6.48	6.76	6.62	6.65	7.63
dp 16	6.07	5.99	6.34	6.11	7.13
dp 17	5.6	5.21	5.81	5.49	6.3
dp 18	5.28	4.78	5.87	5.11	5.98
dp 19	4.99	4.74	6.17	4.94	5.91
dp 20	4.76	4.65	6.07	4.78	5.64
dp 21	4.5	4.46	5.65	4.5	5.26
dp 22	4.16	4.12	5.07	4.2	4.7
dp 23	3.77	3.68	4.59	3.78	4.19
dp 24	3.44	3.36	4.24	3.42	3.75
dp 25	3.08	3.09	3.86	3.07	3.49
dp 26	2.73	2.8	3.36	2.77	3.03
dp 27	2.39	2.58	2.95	2.37	2.65
dp 28	2.07	2.26	2.39	2.01	2.1
dp 29	1.67	1.87	1.87	1.71	1.69
dp 30	1.38	1.58	1.54	1.35	1.3
dp 31	1.07	1.28	1.02	1.04	0.87
dp 32	0.79	0.96	0.7	0.75	0.6
dp 33	0.57	0.69	0.6	0.51	0.51

dp 34	0.36	0.43	0.39	0.32	0.34
dp 35	0.22	0.22	0.19	0.17	0.2
Total	100	100	99.99	100.01	99.98

**Table 9:** The table shows a summary of the contributions of the individual peak areas of the HPAEC chromatogram to the total peak area of wild-type plants (cv. Desiree), of 038VL008 and 038VI107 plants (potato plants with reduced activity of a BEI protein and of an SSIII protein) and of selected lines of the transformations 108CF and 110CF (potato plants with a reduced activity of an SSIII protein and of a BEI protein and of a BEII protein). The number of glucose monomers in the individual side chains is shown as dp.

The peak chain length, whose value is the mean of the two chain lengths (given in DP) which contribute most to the total area under the peaks of the HPAEC chromatogram, is – in the case of debranched amylopectin – of wild-type plants at DP= 13, in the case of 038VL plants likewise at DP= 13 and in the case of the 108CF and 110CF plants, on average, at 15.

If the peak chain length of the transgenic plants is compared with the peak chain length of amylopectin of wild-type plants, the following values result for the peak chain length ratio (PCL ratio):

$$\text{PCL ratio for 038VL} = 13 / 13 = 1$$

$$\text{PCL ratio for 108/110CF} = 15 / 13 = 1.15$$

In addition, the starch granule morphology was analysed using a scanning electron microscope (SEM).

The surface of the starch granules of 108/110CF plants appears coated or raised with pore formation.

Moreover, the granule size determination was carried out using a "Lumosed"-type photosedimentometer from Retsch GmbH, Germany. The mean granule size of untreated starch samples was determined (Table 3).

Mean granule size [ $\mu\text{m}$ ]

Sample	Mean granule size
cv. Desiree	29.7
038VL008	21.5
108CF041	20.8
038VL107	22.9
110CF003	20.7

- 5 **Table 10:** Mean granule size values of starch isolated from wild-type plants (cv. Desiree), plants with a reduced activity of an SSIII and of a BEI protein (038VL008, 038VL107), and of plants with a reduced activity of an SSIII and of a BEI and of a BEII protein (108CF041, 110CF003).

10

### Example 3

#### **Analysis of the amylopectin side chain distribution by means of gel permeation chromatography**

- To separate amylose and amylopectin, 100 mg of starch are dissolved in
- 15 6 ml of 90% strength (v/v) DMSO with constant stirring. After addition of 3 volumes of ethanol, the precipitate is separated off by centrifugation for 10 minutes at 1 800 g at room temperature. The pellet is subsequently washed with 30 ml of ethanol, dried and dissolved in 10 ml of 1% strength (w/v) NaCl solution at 60°C. After cooling the solution to 30°C,
- 20 approximately 50 mg of thymol are added slowly, and this solution is incubated for 2 to 3 days at 30°C. The solution is subsequently centrifuged for 30 minutes at 2 000 g at room temperature. The supernatant is treated with three volumes of ethanol, and the amylopectin which precipitates is separated off by centrifugation for 5 minutes at
- 25 2 000 g at room temperature. The pellet (amylopectin) is washed with

10 ml of 70% strength (v/v) ethanol, centrifuged for 10 minutes at 2 000 g at room temperature and then dried using acetone.

10 mg of amylopectin are subsequently stirred for 10 minutes at 70°C in 250 µl of 90% strength (v/v) DMSO. 375 µl of water at a temperature of 5 80°C are added to the solution until dissolution is complete.

200 µl of this solution are treated with 300 µl of a 16.6 mM sodium acetate solution pH 3.5 and 2 µl of isoamylase (0.24 µl/µl, Megazyme, Sydney, Australia) and the mixture is incubated for 15 hours at 37°C.

A 1:4 dilution of this aqueous isoamylase reaction mixture with DMSO, 10 comprising 90 mM sodium nitrate, is subsequently filtered through a 0.2 µm filter, and 24 µl of the filtrate is analysed chromatographically. Separation was carried out with two columns connected in series, first a Gram PSS3000 (Polymer Standards Service, with suitable precolumn), followed by a Gram PSS100. Detection was by means of refraction index 15 detector (RI 71, Shodex). The column was equilibrated with DMSO comprising 90 mM sodium nitrate. It was eluted with DMSO comprising 90 mM sodium nitrate at a flow rate of 0.7 ml/min over a period of 1 hour.

To correlate the elution volume with the molecular mass, the column used was calibrated with dextran standards. The dextrans used, their molecular 20 mass and the elution volumes are shown in Fig 9. Using the resulting calibration graph, the elution diagram was shown as a molecular weight distribution (Fig 10).

The chromatograms obtained were further evaluated using the program Wingpc Version 6 from Polymer Standards Service GmbH, Mainz, 25 Germany.

The total area under the line of the GPC chromatogram was divided into individual segments, each of which represent groups of side chains of different lengths. The chosen segments contained glucan chains with the following degree of polymerization (DP = number of glucose monomers 30 within one side chain): DP≤11, DP12-18, DP19-24, DP25-30, DP31-36, DP37-42, DP43-48, DP49-55, DP56-61 and DP62-123. To determine the

molecular weight of the individual side chains, a molecular weight of 162 was assumed for glucose. The total area under the line in the GPC chromatogram was then set as 100%, and the percentage of the areas of the individual segments was calculated based on the percentage of the

5 total area. Results obtained from this analysis are shown in Table 11.

	Wild type	08CF041 c
<b>DP<math>\geq</math> 11</b>	100%	40%
<b>DP12-18</b>	100%	50%
<b>DP19-24</b>	100%	69%
<b>DP25-30</b>	100%	91%
<b>DP31-36</b>	100%	111%
<b>DP37-42</b>	100%	116%
<b>DP43-48</b>	100%	110%
<b>DP49-55</b>	100%	107%
<b>DP56-61</b>	100%	109%
<b>DP62-123</b>	100%	157%

**Table 11:** Side chain profiles DP 12 to 18, DP 19 to 24, DP 25 to 30, DP 31 to 36, DP 37 to 42, DP 43-48, DP 49 to 55, DP 56 to 61 and DP 62 to 123 for amylopectin isolated

10 from wild-type plants (cv. Desiree) and from plants with a reduced activity of an SSIII and of a BEI and of a BEII protein (108CF041). The percentages indicate the modification of the individual side chain profiles based on amylopectin isolated from wild-type plants.



**Patent Claims**

1. Plant cell which is genetically modified, the genetic modification  
leading to the reduction of the activity of one or more SSIII proteins  
5 occurring endogenously in the plant cell and to the reduction of the  
activity of one or more BEI proteins which occur endogenously in the  
plant cell and to the reduction of the activity of one or more BEII  
proteins which occur endogenously in the plant cell in comparison to  
corresponding plant cells, of wild-type plants, which have not been  
10 genetically modified.
2. Plant cell according to Claim 1, wherein the genetic modification is the  
introduction of one or more foreign nucleic acid molecules whose  
presence and/or expression leads to the reduction of the activity of one  
15 or more SSIII and BEI and BEII proteins occurring in the plant cell in  
comparison with corresponding plant cells, of wild-type plants, which  
have not been genetically modified.
3. Transgenic plant cells according to Claim 2, wherein said foreign  
20 nucleic acid molecules are selected from the group consisting of
  - a) DNA molecules encoding at least one antisense RNA which brings  
about a reduction of the expression of at least one endogenous  
gene encoding SSIII proteins and/or BEI proteins and/or BEII  
proteins;
  - 25 b) DNA molecules which, via a cosuppression effect, lead to a  
reduction of the expression of at least one endogenous gene  
encoding SSIII protein(s) and/or BEI protein(s) and/or BEII  
protein(s);
  - c) DNA molecules encoding at least one ribozyme which specifically  
30 cleaves transcripts of at least one endogenous gene encoding SSIII  
proteins and/or BEI proteins and/or BEII proteins; and

- 5 d) Nucleic acid molecules introduced by means of in-vivo mutagenesis which lead to a mutation or insertion of a heterologous sequence in at least one endogenous gene encoding SSIII protein(s) and/or BEI protein(s) and/or BEII protein(s), the mutation or insertion bringing about a reduction of the expression of at least one gene encoding SSIII protein(s) and/or BEI protein(s) and/or BEII protein(s), or resulting in the synthesis of inactive SSIII and/or BEI and/or BEII proteins;
- 10 e) DNA molecules which simultaneously encode at least one antisense RNA and at least one sense RNA, where said antisense RNA and said sense RNA form a double-stranded RNA molecule which brings about a reduction of the expression of at least one endogenous gene encoding SSIII protein(s) and/or BEI protein(s) and/or BEII protein(s);
- 15 f) DNA molecules containing transposons, the integration of the transposon sequences leading to a mutation or an insertion in at least one endogenous gene encoding SSIII protein(s) and/or BEI protein(s) and/or BEII protein(s) which brings about a reduction of the expression of at least one gene encoding an SSIII protein(s) and/or BEI protein(s) and/or BEII protein(s), or which results in the synthesis of inactive SSIII and/or BEI and/or BEII proteins; and/or
- 20 g) T-DNA molecules which, owing to the insertion into at least one endogenous gene encoding SSIII protein(s) and/or BEI protein(s) and/or BEII protein(s), bring about a reduction of the expression of at least one gene encoding SSIII protein(s) and/or BEI protein(s) and/or BEII protein(s), or which result in the synthesis of inactive SSIII and/or BEI and/or BEII proteins.
- 25
4. Plant cell according to one of Claims 1 to 3, which synthesizes a modified starch in comparison with a wild-type plant cell which has not been genetically modified.
- 30

5. Plant cell according to Claim 4, wherein the modified starch is characterized in that
- a) it has an amylose content of at least 30%,
  - 5 b) it has an increased phosphate content in comparison with starch from corresponding wild-type plant cells which have not been genetically modified, and
  - c) it has an increased final viscosity in the RVA analysis in comparison with wild-type plant cells which have not been genetically modified.
- 10
6. Plant cell according to Claim 4 or 5, wherein the modified starch is characterized in that it has an increased gel strength in comparison with starch from wild-type plant cells which have not been genetically modified.
- 15
7. Plant cell according to one of Claims 4 to 6, wherein the modified starch is characterized in that it has a modified side chain distribution and/or a modified starch granule morphology in comparison with starch from wild-type plant cells which have not been genetically modified.
- 20
8. Plant containing plant cells according to one of Claims 1 to 7.
9. Method for generating a plant cell which synthesizes a modified starch, comprising the genetic modification of the plant cell, the genetic
- 25 modification leading to the reduction of the activity of one or more SSIII proteins which occur endogenously in the plant cell and to the reduction of the activity of one or more BEI proteins which occur endogenously in the plant cell and to the reduction of the activity of one or more BEII proteins which occur endogenously in the plant cell, in
- 30 comparison with corresponding plant cells, of wild-type plants, which have not been genetically modified.

10. Method for generating a plant cell according to Claim 9 which synthesizes a modified starch, wherein the plant cell is genetically modified by the introduction of one or more foreign nucleic acid molecules whose presence and/or expression leads to the reduction of the activity of in each case at least one SSIII, BEI and BEII protein in comparison with corresponding wild-type plant cells which have not been genetically modified.
11. Method according to Claim 9 or 10, wherein the modified starch is characterized in that
- a) it has an amylose content of at least 30%,
  - b) it has an increased phosphate content in comparison with starch from corresponding wild-type plant cells which have not been genetically modified, and
  - c) it has an increased final viscosity in the RVA analysis in comparison with starch from corresponding wild-type plant cells which have not been genetically modified.
12. Method for generating a genetically modified plant, in which
- a) a plant cell according to one of Claims 9 to 11 is generated;
  - b) a plant is regenerated from, or using, the plant cell generated in accordance with a); and,
  - c) if appropriate, further plants are generated from the plant generated in accordance with step b).
13. Method for generating a transgenic plant according to Claim 12 which synthesizes a modified starch, in which
- a) a plant cell is genetically modified by the introduction of one or more foreign nucleic acid molecules whose presence and/or expression leads to the reduction of the activity of in each case at least one SSIII,

BEI and BEII protein in comparison with corresponding wild-type plant cells which have not been genetically modified;

- b) a plant is regenerated from, or using, the cell generated in accordance with a); and
- 5 c) if appropriate, further plants are generated from the plants generated in accordance with step b).

14. Method according to Claim 12 or 13, wherein the modified starch is characterized in that

- 10 a) it has an amylose content of at least 30%,
- b) it has an increased phosphate content in comparison with starch from corresponding wild-type plant cells which have not been genetically modified, and
- 15 c) it has an increased final viscosity in the RVA analysis in comparison with starch from corresponding wild-type plants which have not been genetically modified.

15. Plant according to Claim 8 or obtainable by the method according to one of Claims 12 to 14, which is a starch-storing plant.

20

16. Plant according to Claim 15, which is a potato plant.

17. Propagation material of plants according to one of Claims 8 or 15 to 16 containing at least one plant cell according to one of Claims 1 to 7.

25

18. Use of one or more nucleic acid molecules which encode proteins with the enzymatic activity of at least one SSIII, at least one BEI and/or at least one BEII protein or their fragments for the generation of plant cells according to one of Claims 1 to 7 or of plants according to one of  
30 Claims 8 or 15 to 16.

19. Use of one or more nucleic acid molecules for the generation of plant cells according to one of Claims 1 to 7 or of plants according to one of Claims 8 or 15 to 16, wherein the foreign nucleic acid molecule(s) are selected from the group consisting of:

- 5 a) DNA molecules encoding at least one antisense RNA which brings about a reduction of the expression of at least one endogenous gene encoding SSIII protein(s) and/or BEI protein(s) and/or BEII protein(s);
- 10 b) DNA molecules which, via a cosuppression effect, lead to a reduction of the expression of at least one endogenous gene encoding SSIII protein(s) and/or BEI protein(s) and/or BEII protein(s);
- 15 c) DNA molecules encoding at least one ribozyme which specifically cleaves transcripts of at least one endogenous gene encoding SSIII protein(s) and/or BEI protein(s) and/or BEII protein(s);
- 20 d) Nucleic acid molecules introduced by means of in-vivo mutagenesis which lead to a mutation or insertion of a heterologous sequence in at least one endogenous gene encoding SSIII protein(s) and/or BEI protein(s) and/or BEII protein(s), the mutation or insertion bringing about a reduction of the expression of at least one gene encoding SSIII protein(s) and/or BEI protein(s) and/or BEII protein(s), or resulting in the synthesis of inactive SSIII and/or BEI and/or BEII proteins;
- 25 e) DNA molecules which simultaneously encode at least one antisense RNA and at least one sense RNA, where said antisense RNA and said sense RNA form a double-stranded RNA molecule which brings about a reduction of the expression of at least one endogenous gene encoding SSIII proteins and/or BEI proteins and/or BEII proteins;
- 30 f) DNA molecules containing transposons, the integration of the transposon sequences leading to a mutation or an insertion in at

- least one endogenous gene encoding SSIII protein(s) and/or BEI protein(s) and/or BEII protein(s) which brings about a reduction of the expression of at least one gene encoding an SSIII protein(s) and/or BEI protein(s) and/or BEII protein(s), or which result in the synthesis of inactive SSIII and/or BEI and/or BEII proteins; and
- 5 g) T-DNA molecules which, owing to the insertion into at least one endogenous gene encoding SSIII proteins and/or BEI proteins and/or BEII proteins, bring about a reduction of the expression of at least one gene encoding SSIII protein(s) and/or BEI protein(s) and/or BEII protein(s), or which result in the synthesis of inactive
- 10 SSIII and/or BEI and/or BEII proteins.

20. Composition comprising at least one of the nucleic acid molecules defined in Claim 19, where the at least one nucleic acid molecule, after
- 15 introduction into a plant cell, leads to the reduction of at least one SSIII protein which occurs endogenously in the plant cell and at least one BEII protein which occurs endogenously in the plant cell and preferably furthermore to the reduction of at least one BEI protein which occurs endogenously in the plant cell.

20

21. Composition according to Claim 20, characterized in that the presence of the at least one nucleic acid molecule in said plant cells leads to a reduction of the activity of in each case at least one SSIII and BEI and BEII protein in comparison with corresponding wild-type plant cells
- 25 which have not been genetically modified.

22. Composition according to one of Claims 20 to 21, the nucleic acid molecules being present in a recombinant nucleic acid molecule.

- 30 23. Use of a composition according to one of Claims 20 to 22, or containing at least one of the nucleic acid molecules defined in Claim

19, for generating a plant cell with a reduced activity of one or more SSIII proteins which occur endogenously in the plant cell and a reduced activity of one or more BEI proteins which occur endogenously in the plant cell and a reduced activity of one or more BEII proteins which occur endogenously in the plant cell, in comparison with corresponding plant cells, of wild-type plants, which have not been genetically modified.

24. Transformation system for plant cells containing at least one nucleic acid molecule and at least one plant cell, the at least one nucleic acid molecule leading to the reduction of the activity of in each case at least one of the SSIII, BEI and BEII proteins which occur endogenously in the plant cell unless the activity of these proteins has been reduced already by an existing genetic modification of said plant cell.

15

25. Host cell containing a composition according to one of Claims 20 to 22.

26. Host cell according to Claim 25, which is a transgenic plant cell containing the composition according to one of Claims 20 to 22.

20

27. Starch which can be obtained from plant cells according to one of Claims 1 to 7 or 26 or from a plant according to one of Claims 8 or 15 to 16 or from propagation material according to Claim 17.

25 28. Starch according to Claim 27, characterized in that

a) it contains amylose content of at least 30%,

b) it has an increased phosphate content in comparison with starch from corresponding wild-type plant cells which have not been genetically modified, and



- c) it has an increased final viscosity in the RVA analysis in comparison with starch from corresponding wild-type plants which have not been genetically modified.
- 5    29. Starch according to Claim 27 or 28, characterized in that it has a modified side chain distribution in comparison with starch from corresponding wild-type plant cells which have not been genetically modified.
- 10   30. Starch according to one of Claims 27 to 29, characterized in that the granule morphology and/or the mean granule size of the starch granules are modified in comparison with wild-type plant cells which have not been genetically modified.
- 15   31. Starch according to one of Claims 27 to 30, which is a potato starch.
- 20   32. Method for producing a starch according to one of Claims 27 to 31, comprising the extraction of the starch from a plant according to one of Claims 8 or 15 to 16 and/or from starch-storing parts of such a plant and/or from a plant cell according to one of Claims 1 to 7 or 26 and/or from propagation material according to Claim 17.
- 25   33. Starch according to one of Claims 27 to 31, obtainable by a method according to Claim 32.
- 30   34. Starch according to one of Claims 27 to 31 or 33, characterized in that it has at least one of the following characteristics:
- a) a final viscosity in the RVA analysis of a 6% (w/w) aqueous starch suspension of at least 300 RVU, preferably at least 400 RVU, in particular at least 500 RVU;

- b) a phosphate content in the C6 position of the glucose monomers of the starch of 40 to 120 nmol, preferably 60 to 100 nmol, in particular 80 to 100 nmol of C6-P per mg of starch;
- c) a porous surface of the starch granules in native form.

5

35. Processing product, in particular foodstuffs or feedstuffs, colour, adhesive, building or insulating material, containing a starch according to one of Claims 27 to 31, 33 or 34.

10 36. Starch-containing part of a plant according to one of Claims 8, 15 or 16.

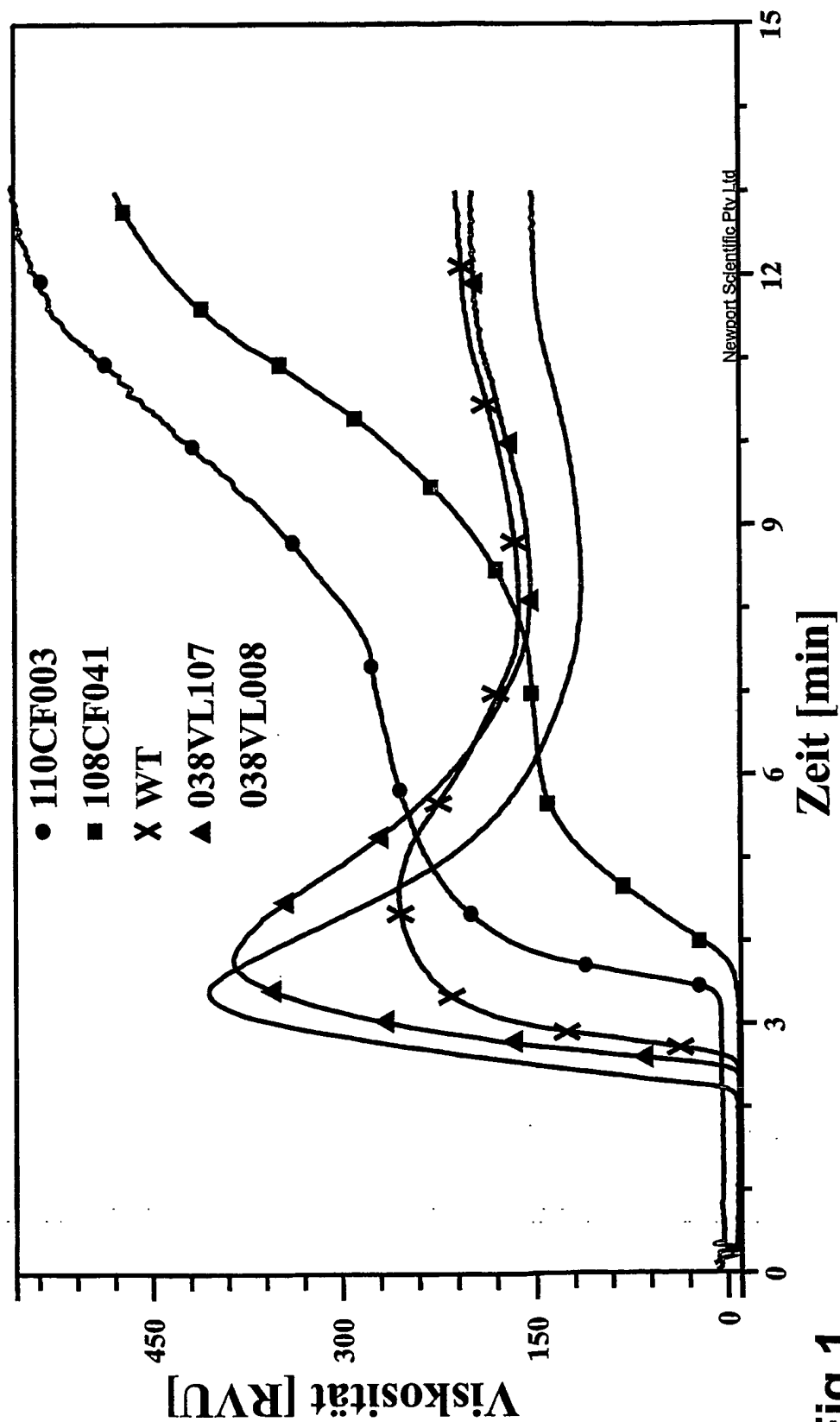
37. Method for modifying the starch of a plant, comprising the method for generating a plant according to one of Claims 12 to 14 and obtaining  
15 starch from the plant or starch-containing parts thereof.

38. Method according to Claim 37, characterized in that the modification of the starch comprises:

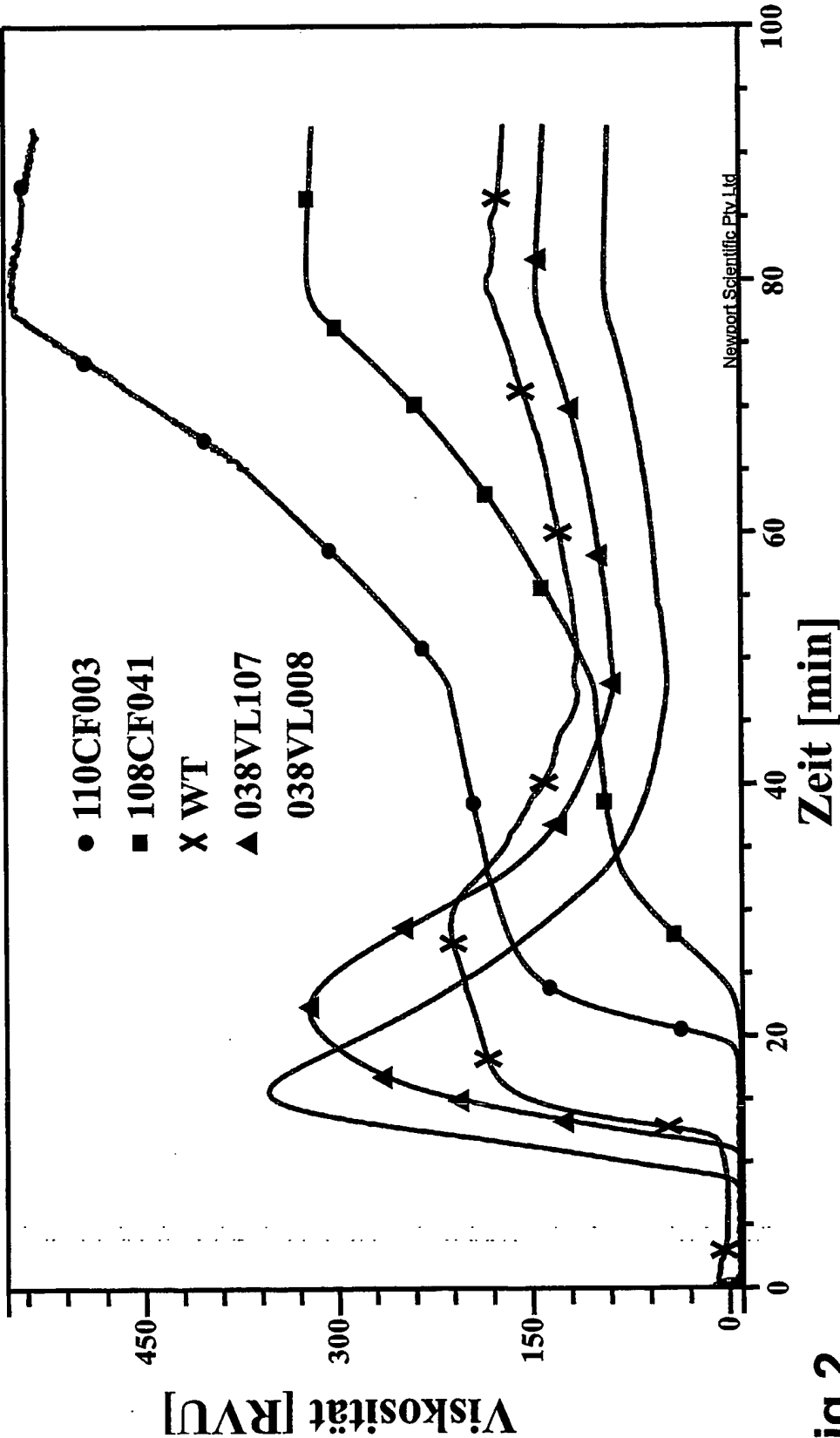
- a) increasing the amylose content of the starch;
- 20 b) increasing the phosphate content of the starch, in particular to a phosphate content in the C6-position of the glucose monomers of the starch of 40 to 120 nmol, preferably 60 to 100 nmol, in particular 80 to 100 nmol of C6-P per mg of starch;
- c) increasing the final viscosity of the starch, in particular to a final  
25 viscosity in the RVA analysis with 6% (w/w) aqueous starch suspension of at least 300 RVU, preferably at least 400 RVU, in particular at least 500 RVU;
- d) increasing the gel strength of the gelatinized starch, and/or
- e) the morphology, in particular the surface structure, porosity and/or  
30 granule size distribution of the native starch granules.

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**RVA-Analysemethode 1****Fig 1**

**RVA-Analysemethode 2**



**Fig 2**

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### RVA-Analysemethode 3

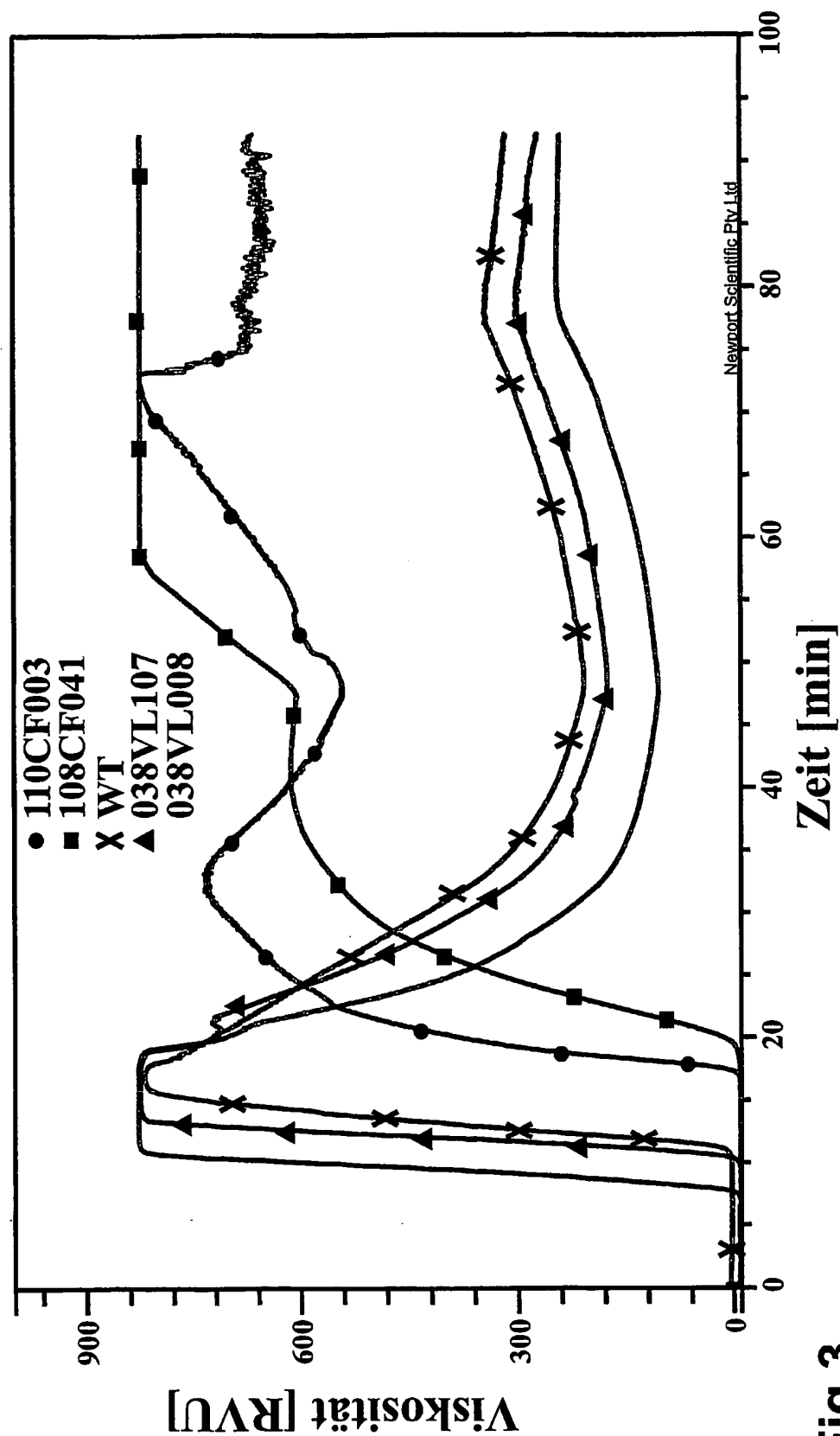
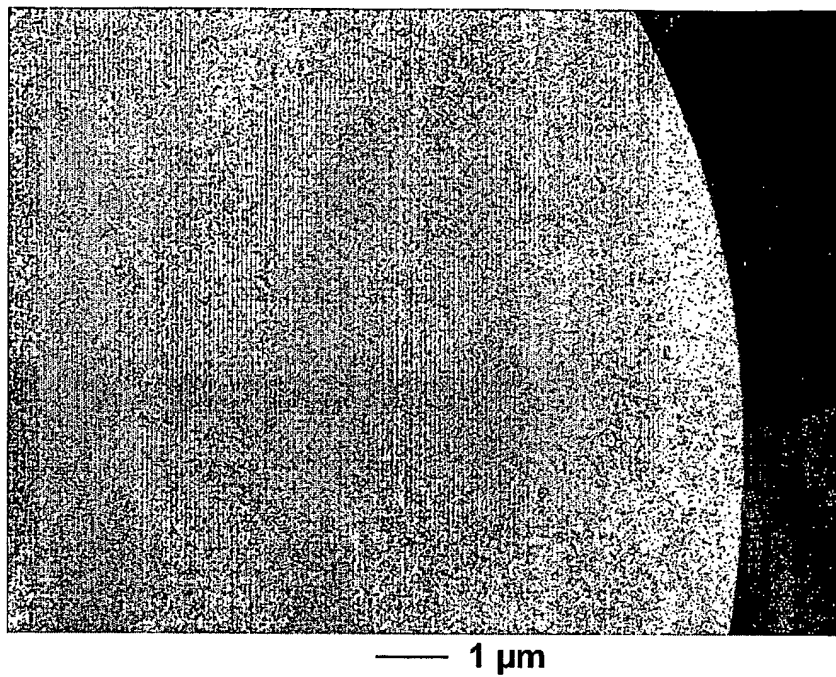
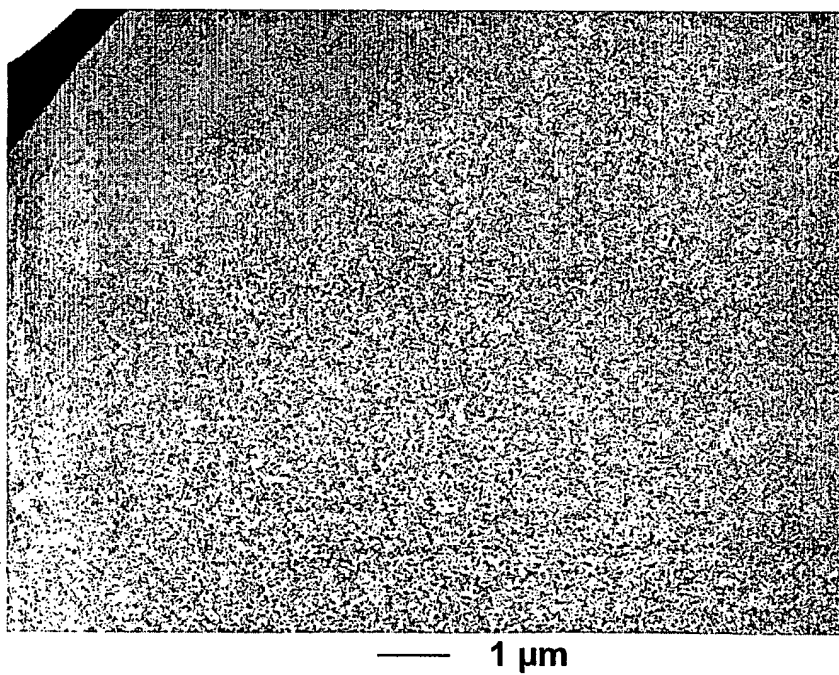


Fig 3



**Fig 4**



**Fig 5**

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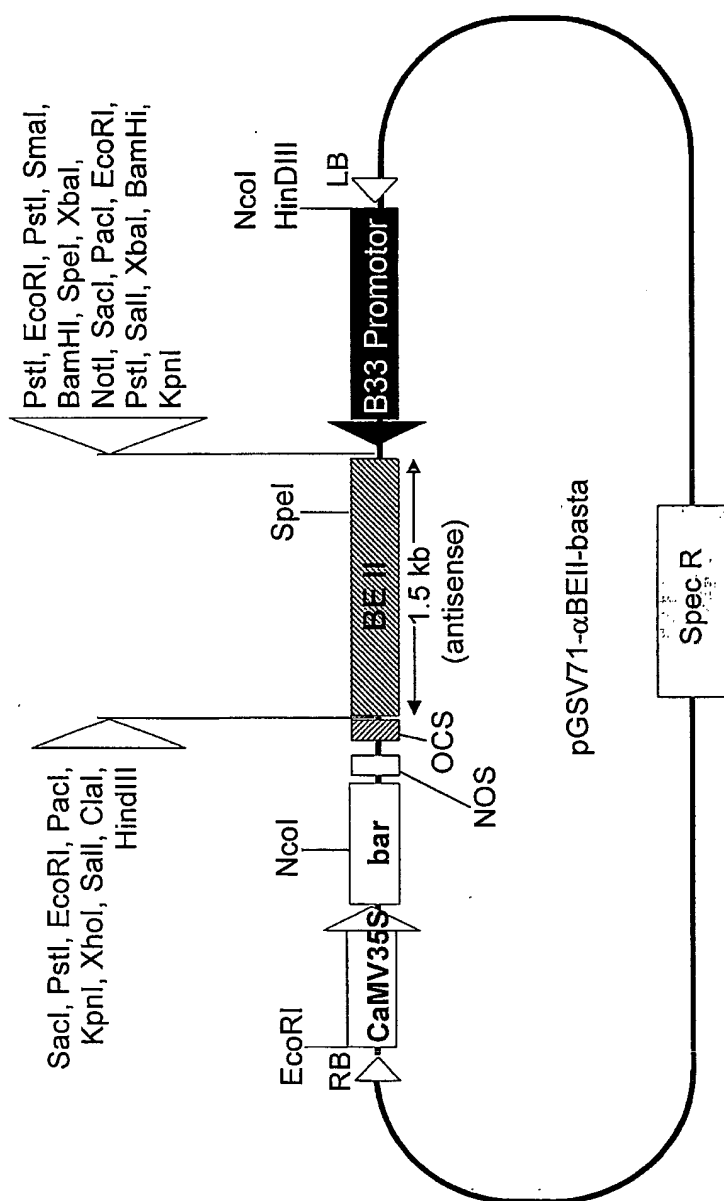


Fig 6

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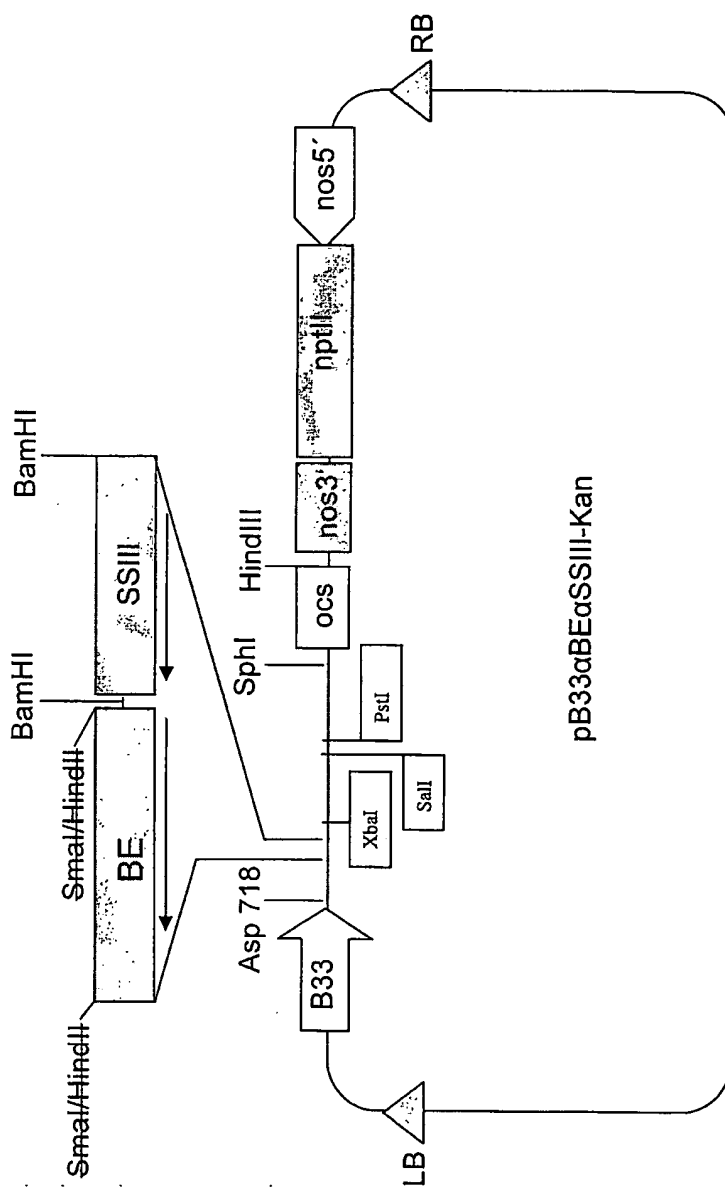


Fig 7



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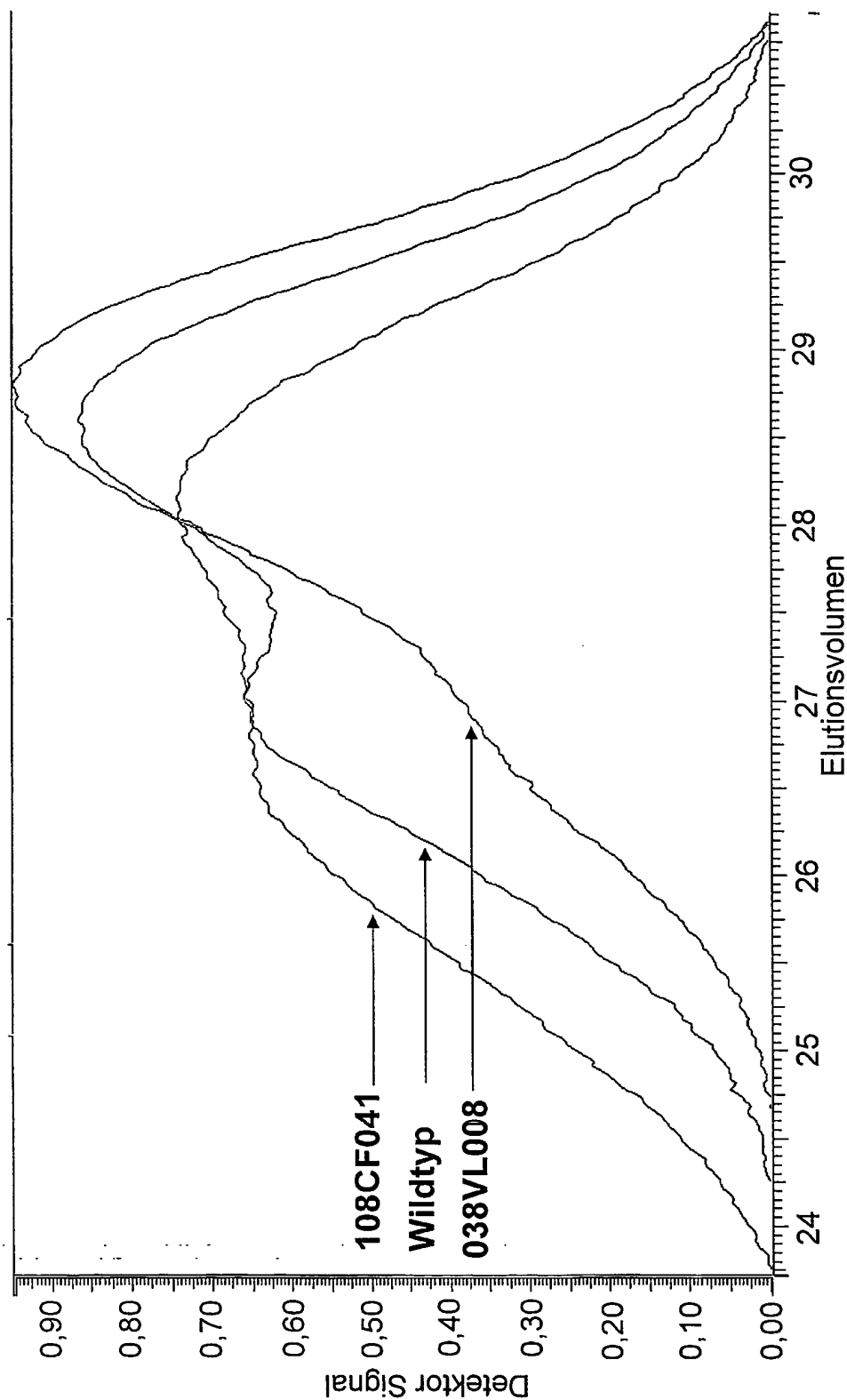
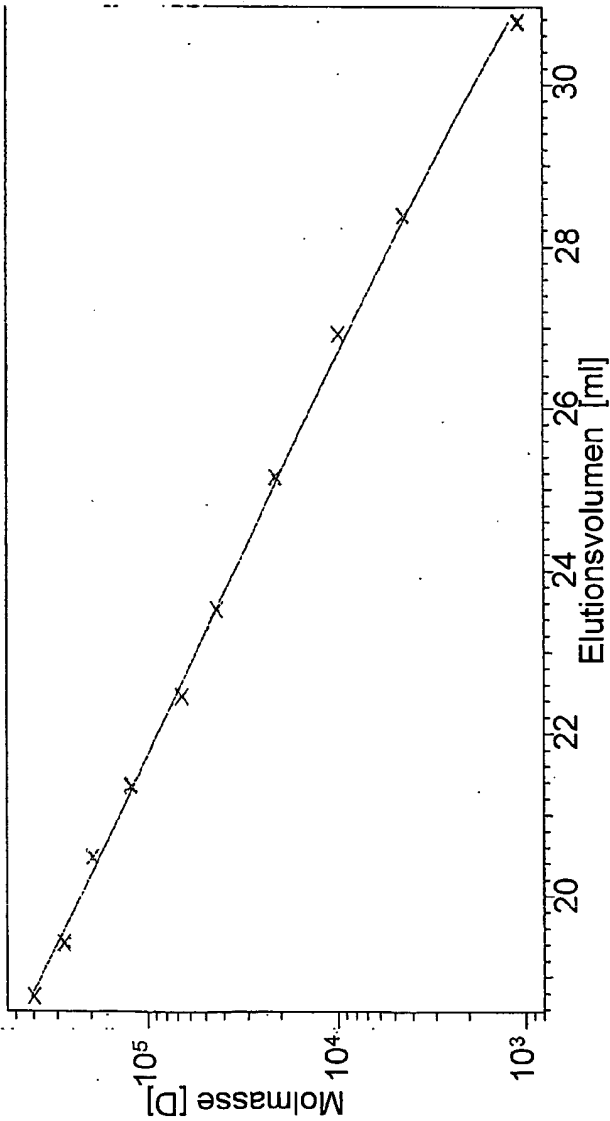


Fig 8

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Kalibriertabelle:

Elutionsvolumen [ml]	Molmasse [D]	Probenname
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19.41	276500	Dextran T410
20.49	196300	Dextran T270
21.35	123600	Dextran T150
22.45	66700	Dextran T80
23.52	43500	Dextran T50
25.15	21400	Dextran T25
26.92	9890	Dextran T12
28.38	4440	Dextran T5
30.77	1080	Dextran T1

Fig 9

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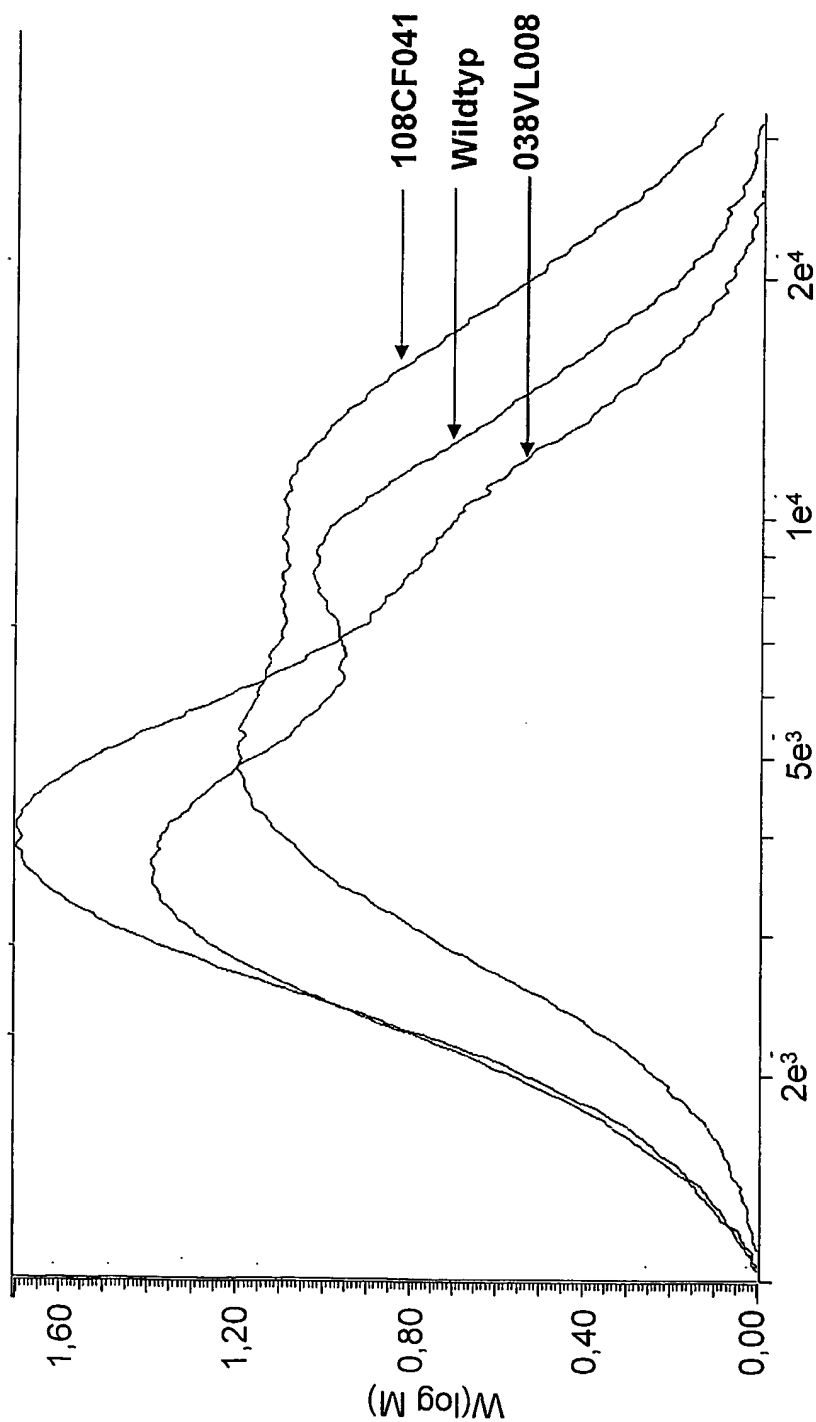


Fig 10

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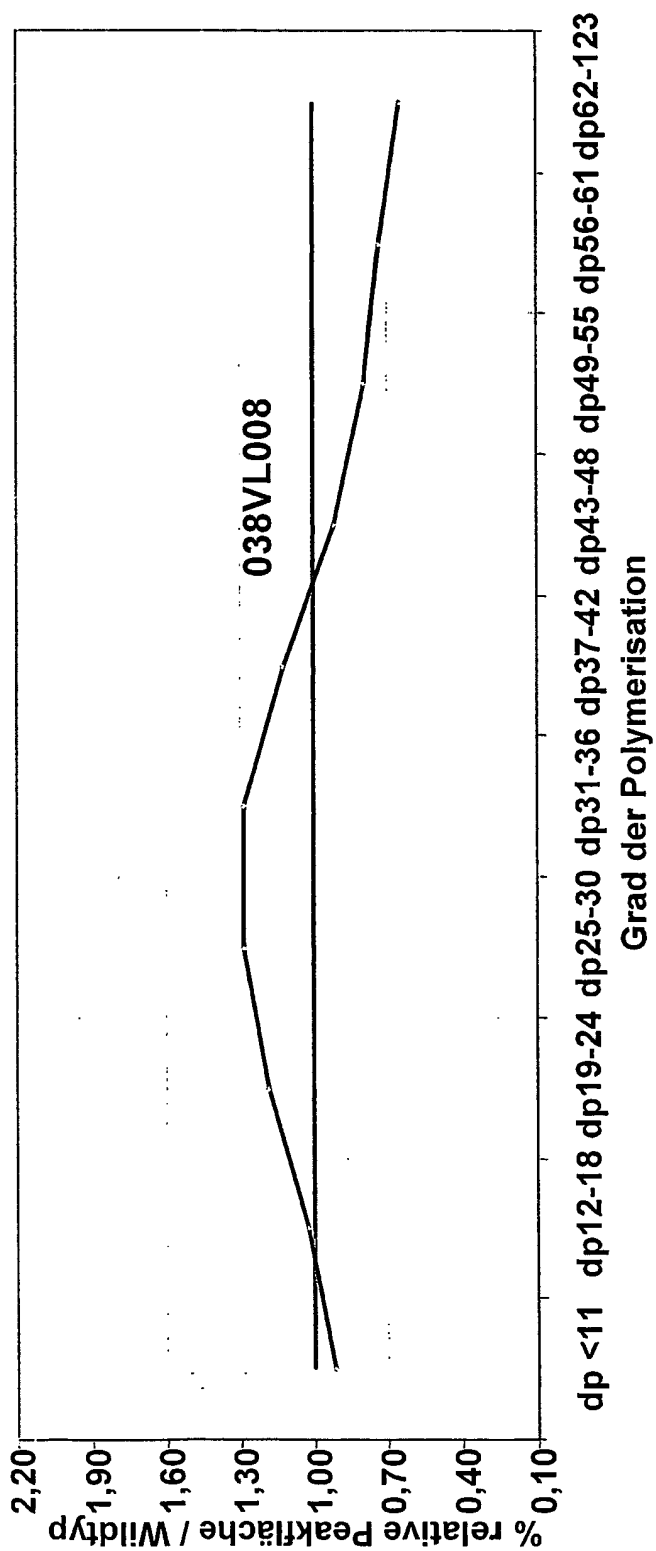


Fig 11

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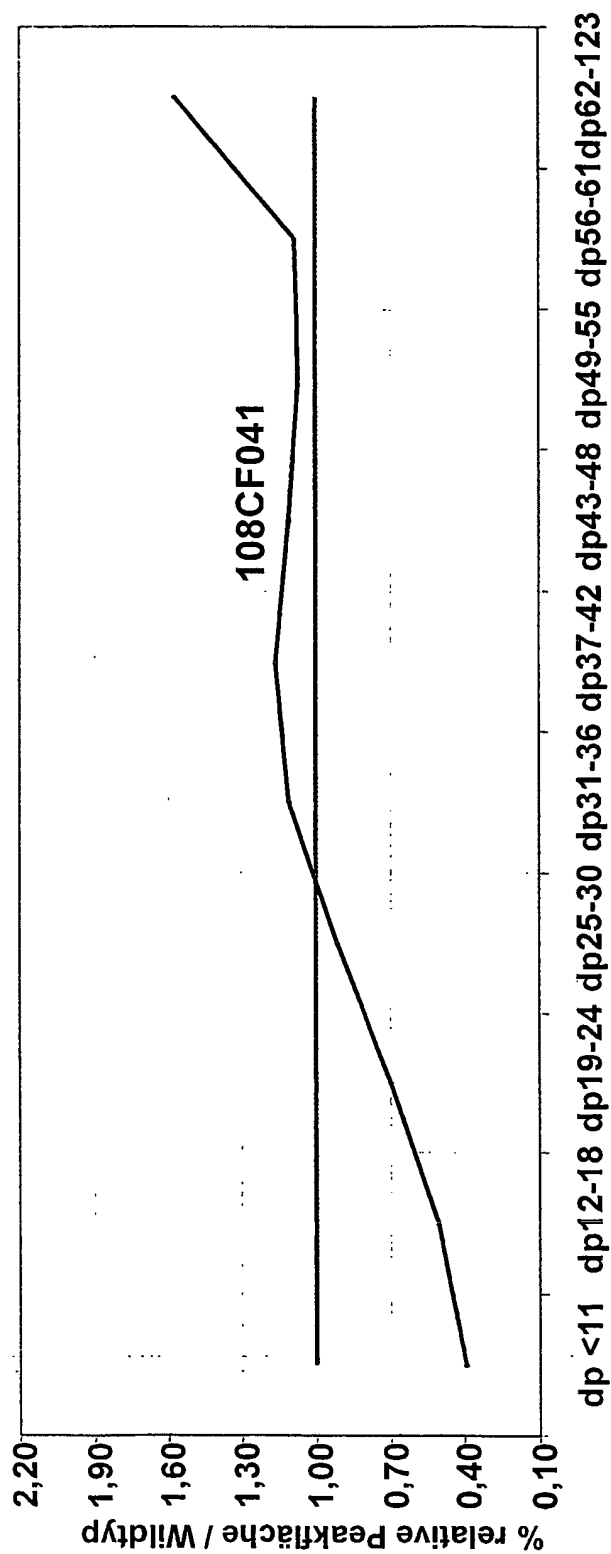


Fig. 12

## SEQUENCE LISTING

10/539723  
JC17 Rec'd PCT/PTO 20 JUN 2005

<110> Bayer CropScience GmbH

<120> Plant cells and plants which synthesize a starch with an increased final viscosity

<130> BCS 02 5002 - PCT

<150> EP 02028530.0

<151> 2002-12-19

<150> EP 03090275.3

<151> 2003-08-29

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<301> Abel, G.J., Springer, F., Willmitzer, L. and Kossmann, J.

<302> Cloning and functional analysis of a cDNA encoding a novel  
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<303> Plant J.

<304> 10

<305> 6

<306> 981-991

<307> 1996

<308> X94400

<309> 1995-12-22

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233

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5

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10

15

20

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30

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75

80

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90

95

100

105

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569

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110

115

120



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220

225

230

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 Ala Asn Leu Arg Arg Gln Ala Ile Glu Arg Leu Ala Glu Glu Asn Leu  
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 ttg caa ggg atc aga tta ttt tgt ttt cca gag gtt gta aaa cct gat  
 1241  
 Leu Gln Gly Ile Arg Leu Phe Cys Phe Pro Glu Val Val Lys Pro Asp  
 330 335 340 345  
  
 gaa gat gtc gag ata ttt ctt aac aga ggt ctt tcc act ttg aag aat  
 1289  
 Glu Asp Val Glu Ile Phe Leu Asn Arg Gly Leu Ser Thr Leu Lys Asn

350	355	360
gag tct gat gtc ttg att atg gga gct ttt aat gag tgg cgc tat agg		
1337		
Glu Ser Asp Val Leu Ile Met Gly Ala Phe Asn Glu Trp Arg Tyr Arg		
365	370	375
tct ttt act aca agg cta act gag act cat ctc aat gga gat tgg tgg		
1385		
Ser Phe Thr Thr Arg Leu Thr Glu Thr His Leu Asn Gly Asp Trp Trp		
380	385	390
tct tgc aag atc cat gtt ccc aag gaa gca tac agg gct gat ttt gtg		
1433		
Ser Cys Lys Ile His Val Pro Lys Glu Ala Tyr Arg Ala Asp Phe Val		
395	400	405
ttt ttt aat gga caa gat gtc tat gac aac aat gat gga aat gac ttc		
1481		
Phe Phe Asn Gly Gln Asp Val Tyr Asp Asn Asn Asp Gly Asn Asp Phe		
410	415	420
425		
agt ata act gtg aaa ggt ggt atg caa atc att gac ttt gaa aat ttc		
1529		
Ser Ile Thr Val Lys Gly Gly Met Gln Ile Ile Asp Phe Glu Asn Phe		
430	435	440
ttg ctt gag gag aaa tgg aga gaa cag gag aaa ctt gct aaa gaa caa		
1577		
Leu Leu Glu Glu Lys Trp Arg Glu Gln Glu Lys Leu Ala Lys Glu Gln		
445	450	455
gct gaa aga gaa aga cta gcg gaa gaa caa aga cga ata gaa gca gag		
1625		
Ala Glu Arg Glu Arg Leu Ala Glu Glu Gln Arg Arg Ile Glu Ala Glu		
460	465	470

aaa gct gaa att gaa gct gac aga gca caa gca aag gaa gag gct gca  
1673

Lys Ala Glu Ile Glu Ala Asp Arg Ala Gln Ala Lys Glu Glu Ala Ala

475

480

485

aag aaa aag aaa gta ttg cga gaa ttg atg gta aaa gcc acg aag act  
1721

Lys Lys Lys Lys Val Leu Arg Glu Leu Met Val Lys Ala Thr Lys Thr

490

495

500

505

cgt gat atc acg tgg tac ata gag cca agt gaa ttt aaa tgc gag gac  
1769

Arg Asp Ile Thr Trp Tyr Ile Glu Pro Ser Glu Phe Lys Cys Glu Asp

510

515

520

aag gtc agg tta tac tat aac aaa agt tca ggt cct ctc tcc cat gct  
1817

Lys Val Arg Leu Tyr Tyr Asn Lys Ser Ser Gly Pro Leu Ser His Ala

525

530

535

aag gac ttg tgg atc cac gga gga tat aat aat tgg aag gat ggt ttg  
1865

Lys Asp Leu Trp Ile His Gly Gly Tyr Asn Asn Trp Lys Asp Gly Leu

540

545

550

tct att gtc aaa aag ctt gtt aaa tct gag aga ata gat ggt gat tgg  
1913

Ser Ile Val Lys Lys Leu Val Lys Ser Glu Arg Ile Asp Gly Asp Trp

555

560

565

tgg tat aca gag gtt gtt att cct gat cag gca ctt ttc ttg gat tgg  
1961

Trp Tyr Thr Glu Val Val Ile Pro Asp Gln Ala Leu Phe Leu Asp Trp

570

575

580

585

gtt ttt gct gat ggt cca ccc aag cat gcc att gct tat gat aac aat  
2009

Val Phe Ala Asp Gly Pro Pro Lys His Ala Ile Ala Tyr Asp Asn Asn

	590	595	600
cac cgc caa gac ttc cat gcc att gtc ccc aac cac att ccg gag gaa 2057			
His Arg Gln Asp Phe His Ala Ile Val Pro Asn His Ile Pro Glu Glu			
605	610	615	
tta tat tgg gtt gag gaa gaa cat cag atc ttt aag aca ctt cag gag 2105			
Leu Tyr Trp Val Glu Glu Glu His Gln Ile Phe Lys Thr Leu Gln Glu			
620	625	630	
gag aga agg ctt aga gaa gcg gct atg cgt gct aag gtt gaa aaa aca 2153			
Glu Arg Arg Leu Arg Glu Ala Ala Met Arg Ala Lys Val Glu Lys Thr			
635	640	645	
gca ctt ctg aaa act gaa aca aag gaa aga act atg aaa tca ttt tta 2201			
Ala Leu Leu Lys Thr Glu Thr Lys Glu Arg Thr Met Lys Ser Phe Leu			
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ctg tct cag aag cat gta gta tat act gag cct ctt gat atc caa gct 2249			
Leu Ser Gln Lys His Val Val Tyr Thr Glu Pro Leu Asp Ile Gln Ala			
670	675	680	
gga agc agc gtc aca gtt tac tat aat ccc gcc aat aca gta ctt aat 2297			
Gly Ser Ser Val Thr Val Tyr Tyr Asn Pro Ala Asn Thr Val Leu Asn			
685	690	695	
ggt aaa cct gaa att tgg ttc aga tgt tca ttt aat cgc tgg act cac 2345			
Gly Lys Pro Glu Ile Trp Phe Arg Cys Ser Phe Asn Arg Trp Thr His			
700	705	710	

cgc ctg ggt cca ttg cca cct cag aaa atg tcg cct gct gaa aat ggc  
2393

Arg Leu Gly Pro Leu Pro Pro Gln Lys Met Ser Pro Ala Glu Asn Gly

715

720

725

acc cat gtc aga gca act gtg aag gtt cca ttg gat gca tat atg atg  
2441

Thr His Val Arg Ala Thr Val Lys Val Pro Leu Asp Ala Tyr Met Met

730

735

740

745

gat ttt gta ttt tcc gag aga gaa gat ggt ggg att ttt gac aat aag  
2489

Asp Phe Val Phe Ser Glu Arg Glu Asp Gly Gly Ile Phe Asp Asn Lys

750

755

760

agc gga atg gac tat cac ata cct gtg ttt gga gga gtc gct aaa gaa  
2537

Ser Gly Met Asp Tyr His Ile Pro Val Phe Gly Gly Val Ala Lys Glu

765

770

775

cct cca atg cat att gtc cat att gct gtc gaa atg gca cca att gca  
2585

Pro Pro Met His Ile Val His Ile Ala Val Glu Met Ala Pro Ile Ala

780

785

790

aag gtg gga ggc ctt ggt gat gtt gtt act agt ctt tcc cgt gct gtt  
2633

Lys Val Gly Gly Leu Gly Asp Val Val Thr Ser Leu Ser Arg Ala Val

795

800

805

caa gat tta aac cat aat gtg gat att atc tta cct aag tat gac tgt  
2681

Gln Asp Leu Asn His Asn Val Asp Ile Ile Leu Pro Lys Tyr Asp Cys

810

815

820

825

ttg aag atg aat aat gtg aag gac ttt cgg ttt cac aaa aac tac ttt  
2729

Leu Lys Met Asn Asn Val Lys Asp Phe Arg Phe His Lys Asn Tyr Phe

830

835

840

tgg ggt ggg act gaa ata aaa gta tgg ttt gga aag gtg gaa ggt ctc  
2777

Trp Gly Gly Thr Glu Ile Lys Val Trp Phe Gly Lys Val Glu Gly Leu

845

850

855

tcg gtc tat ttt ttg gag cct caa aac ggg tta ttt tcg aaa ggg tgc  
2825

Ser Val Tyr Phe Leu Glu Pro Gln Asn Gly Leu Phe Ser Lys Gly Cys

860

865

870

gtc tat ggt tgt agc aat gat ggt gaa cga ttt ggt ttc ttc tgt cac  
2873

Val Tyr Gly Cys Ser Asn Asp Gly Glu Arg Phe Gly Phe Phe Cys His

875

880

885

gcg gct ttg gag ttt ctt ctg caa ggt gga ttt agt ccg gat atc att  
2921

Ala Ala Leu Glu Phe Leu Leu Gln Gly Gly Phe Ser Pro Asp Ile Ile

890

895

900

905

cat tgc cat gat tgg tct agt gct cct gtt gct tgg ctc ttt aag gaa  
2969

His Cys His Asp Trp Ser Ser Ala Pro Val Ala Trp Leu Phe Lys Glu

910

915

920

caa tat aca cac tat ggt cta agc aaa tct cgt ata gtc ttc acg ata  
3017

Gln Tyr Thr His Tyr Gly Leu Ser Lys Ser Arg Ile Val Phe Thr Ile

925

930

935

cat aat ctt gaa ttt ggg gca gat ctc att ggg aga gca atg act aac  
3065

His Asn Leu Glu Phe Gly Ala Asp Leu Ile Gly Arg Ala Met Thr Asn

940

945

950

gca gac aaa gct aca aca gtt tca cca act tac tca cag gag gtg tct  
3113

Ala Asp Lys Ala Thr Thr Val Ser Pro Thr Tyr Ser Gln Glu Val Ser

955

960

965

gga aac cct gta att gcg cct cac ctt cac aag ttc cat ggt ata gtg  
3161

Gly Asn Pro Val Ile Ala Pro His Leu His Lys Phe His Gly Ile Val

970

975

980

985

aat ggg att gac cca gat att tgg gat cct tta aac gat aag ttc att  
3209

Asn Gly Ile Asp Pro Asp Ile Trp Asp Pro Leu Asn Asp Lys Phe Ile

990

995

1000

ccg att ccg tac acc tca gaa aac gtt gtt gaa ggc aaa aca gca  
3254

Pro Ile Pro Tyr Thr Ser Glu Asn Val Val Glu Gly Lys Thr Ala

1005

1010

1015

gcc aag gaa gct ttg cag cga aaa ctt gga ctg aaa cag gct gac  
3299

Ala Lys Glu Ala Leu Gln Arg Lys Leu Gly Leu Lys Gln Ala Asp

1020

1025

1030

ctt cct ttg gta gga att atc acc cgc tta act cac cag aaa gga  
3344

Leu Pro Leu Val Gly Ile Ile Thr Arg Leu Thr His Gln Lys Gly

1035

1040

1045

atc cac ctc att aaa cat gct att tgg cgc acc ttg gaa cgg aac  
3389

Ile His Leu Ile Lys His Ala Ile Trp Arg Thr Leu Glu Arg Asn

1050

1055

1060

gga cag gta gtc ttg ctt ggt tct gct cct gat cct agg gta caa



3434  
 Gly Gln Val Val Leu Leu Gly Ser Ala Pro Asp Pro Arg Val Gln  
 1065 1070 1075  
  
 aac gat ttt gtt aat ttg gca aat caa ttg cac tcc aaa tat aat  
 3479  
 Asn Asp Phe Val Asn Leu Ala Asn Gln Leu His Ser Lys Tyr Asn  
 1080 1085 1090  
  
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 3524  
 Asp Arg Ala Arg Leu Cys Leu Thr Tyr Asp Glu Pro Leu Ser His  
 1095 1100 1105  
  
 ctg ata tat gct ggt gct gat ttt att cta gtt cct tca ata ttt  
 3569  
 Leu Ile Tyr Ala Gly Ala Asp Phe Ile Leu Val Pro Ser Ile Phe  
 1110 1115 1120  
  
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 3614  
 Glu Pro Cys Gly Leu Thr Gln Leu Thr Ala Met Arg Tyr Gly Ser  
 1125 1130 1135  
  
 att cca gtc gtg cgt aaa act gga gga ctt tat gat act gta ttt  
 3659  
 Ile Pro Val Val Arg Lys Thr Gly Gly Leu Tyr Asp Thr Val Phe  
 1140 1145 1150  
  
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 3704  
 Asp Val Asp His Asp Lys Glu Arg Ala Gln Gln Cys Gly Leu Glu  
 1155 1160 1165  
  
 cca aat gga ttc agc ttt gat gga gca gat gct ggc gga gtt gat  
 3749  
 Pro Asn Gly Phe Ser Phe Asp Gly Ala Asp Ala Gly Gly Val Asp

1170	1175	1180
tat gct ctg aat	aga gct ctc tct gct	tgg tac gat ggt cgg gat
3794		
Tyr Ala Leu Asn	Arg Ala Leu Ser Ala	Trp Tyr Asp Gly Arg Asp
1185	1190	1195
tgg ttc aac tct	tta tgc aag cag gtc	atg gaa caa gat tgg tct
3839		
Trp Phe Asn Ser	Leu Cys Lys Gln Val	Met Glu Gln Asp Trp Ser
1200	1205	1210
tgg aac cga cct	gct ctt gat tat ttg	gag ctt tac cat gct gct
3884		
Trp Asn Arg Pro	Ala Leu Asp Tyr Leu	Glu Leu Tyr His Ala Ala
1215	1220	1225
aga aag tta gaa	tag ttagtttgtg	agatgctagc agaaaaattc acgagatctg
3939		
Arg Lys Leu Glu		
1230		
caatctgtac aggttcagtg tttgcgtctg gacagctttt ttatttccta tatcaaagta		
3999		
taaatacaagt ctacactgag atcaatagca gacagtcctc agttcatttc attttttgtg		
4059		
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<212> PRT		
<213> Solanum tuberosum		

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Ser Asn Ala Ile Thr His Leu Lys Ile Lys Pro Phe Leu Gly Phe Val  
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Ser His Gly Thr Thr Ser Leu Ser Val Gln Ser Ser Ser Trp Arg Lys  
 35 40 45

Asp Gly Met Val Thr Gly Val Ser Phe Pro Phe Cys Ala Asn Leu Ser  
 50 55 60

Gly Arg Arg Arg Arg Lys Val Ser Thr Thr Arg Ser Gln Gly Ser Ser  
 65 70 75 80

Pro Lys Gly Phe Val Pro Arg Lys Pro Ser Gly Met Ser Thr Gln Arg  
 85 90 95

Lys Val Gln Lys Ser Asn Gly Asp Lys Glu Ser Gln Ser Thr Ser Thr  
 100 105 110

Ser Lys Glu Ser Glu Ile Ser Asn Gln Lys Thr Val Glu Ala Arg Val  
 115 120 125

Glu Thr Ser Asp Asp Asp Thr Lys Val Val Val Arg Asp His Lys Phe  
 130 135 140

Leu Glu Asp Glu Asp Glu Ile Asn Gly Ser Thr Lys Ser Ile Ser Met  
 145 150 155 160

Ser Pro Val Arg Val Ser Ser Gln Phe Val Glu Ser Glu Glu Thr Gly  
 165 170 175

Gly Asp Asp Lys Asp Ala Val Lys Leu Asn Lys Ser Lys Arg Ser Glu  
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Glu Ser Asp Phe Leu Ile Asp Ser Val Ile Arg Glu Gln Ser Gly Ser  
 195 200 205

Gln Gly Glu Thr Asn Ala Ser Ser Lys Gly Ser His Ala Val Gly Thr  
 210 215 220

Lys Leu Tyr Glu Ile Leu Gln Val Asp Val Glu Pro Gln Gln Leu Lys  
 225 230 235 240

Glu Asn Asn Ala Gly Asn Val Glu Tyr Lys Gly Pro Val Ala Ser Lys  
 245 250 255

Leu Leu Glu Ile Thr Lys Ala Ser Asp Val Glu His Thr Glu Ser Asn  
 260 265 270

Glu Ile Asp Asp Leu Asp Thr Asn Ser Phe Phe Lys Ser Asp Leu Ile  
 275 280 285

Glu Glu Asp Glu Pro Leu Ala Ala Gly Thr Val Glu Thr Gly Asp Ser  
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Ser Leu Asn Leu Arg Leu Glu Met Glu Ala Asn Leu Arg Arg Gln Ala  
 305 310 315 320

Ile Glu Arg Leu Ala Glu Glu Asn Leu Leu Gln Gly Ile Arg Leu Phe  
 325 330 335

Cys Phe Pro Glu Val Val Lys Pro Asp Glu Asp Val Glu Ile Phe Leu  
 340 345 350

Asn Arg Gly Leu Ser Thr Leu Lys Asn Glu Ser Asp Val Leu Ile Met  
 355 360 365

Gly Ala Phe Asn Glu Trp Arg Tyr Arg Ser Phe Thr Thr Arg Leu Thr  
 370 375 380

Glu Thr His Leu Asn Gly Asp Trp Trp Ser Cys Lys Ile His Val Pro  
 385 390 395 400

Lys Glu Ala Tyr Arg Ala Asp Phe Val Phe Phe Asn Gly Gln Asp Val  
 405 410 415  
 Tyr Asp Asn Asn Asp Gly Asn Asp Phe Ser Ile Thr Val Lys Gly Gly  
 420 425 430  
 Met Gln Ile Ile Asp Phe Glu Asn Phe Leu Leu Glu Glu Lys Trp Arg  
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 Glu Gln Glu Lys Leu Ala Lys Glu Gln Ala Glu Arg Glu Arg Leu Ala  
 450 455 460  
 Glu Glu Gln Arg Arg Ile Glu Ala Glu Lys Ala Glu Ile Glu Ala Asp  
 465 470 475 480  
 Arg Ala Gln Ala Lys Glu Glu Ala Ala Lys Lys Lys Lys Val Leu Arg  
 485 490 495  
 Glu Leu Met Val Lys Ala Thr Lys Thr Arg Asp Ile Thr Trp Tyr Ile  
 500 505 510  
 Glu Pro Ser Glu Phe Lys Cys Glu Asp Lys Val Arg Leu Tyr Tyr Asn  
 515 520 525  
 Lys Ser Ser Gly Pro Leu Ser His Ala Lys Asp Leu Trp Ile His Gly  
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 Gly Tyr Asn Asn Trp Lys Asp Gly Leu Ser Ile Val Lys Lys Leu Val  
 545 550 555 560  
 Lys Ser Glu Arg Ile Asp Gly Asp Trp Trp Tyr Thr Glu Val Val Ile  
 565 570 575  
 Pro Asp Gln Ala Leu Phe Leu Asp Trp Val Phe Ala Asp Gly Pro Pro  
 580 585 590  
 Lys His Ala Ile Ala Tyr Asp Asn Asn His Arg Gln Asp Phe His Ala  
 595 600 605

Ile Val Pro Asn His Ile Pro Glu Glu Leu Tyr Trp Val Glu Glu Glu  
 610 615 620

His Gln Ile Phe Lys Thr Leu Gln Glu Glu Arg Arg Leu Arg Glu Ala  
 625 630 635 640

Ala Met Arg Ala Lys Val Glu Lys Thr Ala Leu Leu Lys Thr Glu Thr  
 645 650 655

Lys Glu Arg Thr Met Lys Ser Phe Leu Leu Ser Gln Lys His Val Val  
 660 665 670

Tyr Thr Glu Pro Leu Asp Ile Gln Ala Gly Ser Ser Val Thr Val Tyr  
 675 680 685

Tyr Asn Pro Ala Asn Thr Val Leu Asn Gly Lys Pro Glu Ile Trp Phe  
 690 695 700

Arg Cys Ser Phe Asn Arg Trp Thr His Arg Leu Gly Pro Leu Pro Pro  
 705 710 715 720

Gln Lys Met Ser Pro Ala Glu Asn Gly Thr His Val Arg Ala Thr Val  
 725 730 735

Lys Val Pro Leu Asp Ala Tyr Met Met Asp Phe Val Phe Ser Glu Arg  
 740 745 750

Glu Asp Gly Gly Ile Phe Asp Asn Lys Ser Gly Met Asp Tyr His Ile  
 755 760 765

Pro Val Phe Gly Gly Val Ala Lys Glu Pro Pro Met His Ile Val His  
 770 775 780

Ile Ala Val Glu Met Ala Pro Ile Ala Lys Val Gly Gly Leu Gly Asp  
 785 790 795 800

Val Val Thr Ser Leu Ser Arg Ala Val Gln Asp Leu Asn His Asn Val  
 805 810 815

Asp Ile Ile Leu Pro Lys Tyr Asp Cys Leu Lys Met Asn Asn Val Lys  
 820 825 830

Asp Phe Arg Phe His Lys Asn Tyr Phe Trp Gly Gly Thr Glu Ile Lys  
 835 840 845

Val Trp Phe Gly Lys Val Glu Gly Leu Ser Val Tyr Phe Leu Glu Pro  
 850 855 860

Gln Asn Gly Leu Phe Ser Lys Gly Cys Val Tyr Gly Cys Ser Asn Asp  
 865 870 875 880

Gly Glu Arg Phe Gly Phe Phe Cys His Ala Ala Leu Glu Phe Leu Leu  
 885 890 895

Gln Gly Gly Phe Ser Pro Asp Ile Ile His Cys His Asp Trp Ser Ser  
 900 905 910

Ala Pro Val Ala Trp Leu Phe Lys Glu Gln Tyr Thr His Tyr Gly Leu  
 915 920 925

Ser Lys Ser Arg Ile Val Phe Thr Ile His Asn Leu Glu Phe Gly Ala  
 930 935 940

Asp Leu Ile Gly Arg Ala Met Thr Asn Ala Asp Lys Ala Thr Thr Val  
 945 950 955 960

Ser Pro Thr Tyr Ser Gln Glu Val Ser Gly Asn Pro Val Ile Ala Pro  
 965 970 975

His Leu His Lys Phe His Gly Ile Val Asn Gly Ile Asp Pro Asp Ile  
 980 985 990

Trp Asp Pro Leu Asn Asp Lys Phe Ile Pro Ile Pro Tyr Thr Ser Glu  
 995 1000 1005

Asn Val Val Glu Gly Lys Thr Ala Ala Lys Glu Ala Leu Gln Arg  
 1010 1015 1020

Lys	Leu	Gly	Leu	Lys	Gln	Ala	Asp	Leu	Pro	Leu	Val	Gly	Ile	Ile
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Thr	Arg	Leu	Thr	His	Gln	Lys	Gly	Ile	His	Leu	Ile	Lys	His	Ala
1040						1045					1050			
Ile	Trp	Arg	Thr	Leu	Glu	Arg	Asn	Gly	Gln	Val	Val	Leu	Leu	Gly
1055						1060					1065			
Ser	Ala	Pro	Asp	Pro	Arg	Val	Gln	Asn	Asp	Phe	Val	Asn	Leu	Ala
1070						1075					1080			
Asn	Gln	Leu	His	Ser	Lys	Tyr	Asn	Asp	Arg	Ala	Arg	Leu	Cys	Leu
1085						1090					1095			
Thr	Tyr	Asp	Glu	Pro	Leu	Ser	His	Leu	Ile	Tyr	Ala	Gly	Ala	Asp
1100						1105					1110			
Phe	Ile	Leu	Val	Pro	Ser	Ile	Phe	Glu	Pro	Cys	Gly	Leu	Thr	Gln
1115						1120					1125			
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1130						1135					1140			
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1145						1150					1155			
Arg	Ala	Gln	Gln	Cys	Gly	Leu	Glu	Pro	Asn	Gly	Phe	Ser	Phe	Asp
1160						1165					1170			
Gly	Ala	Asp	Ala	Gly	Gly	Val	Asp	Tyr	Ala	Leu	Asn	Arg	Ala	Leu
1175						1180					1185			
Ser	Ala	Trp	Tyr	Asp	Gly	Arg	Asp	Trp	Phe	Asn	Ser	Leu	Cys	Lys
1190						1195					1200			
Gln	Val	Met	Glu	Gln	Asp	Trp	Ser	Trp	Asn	Arg	Pro	Ala	Leu	Asp
1205						1210					1215			



Tyr Leu Glu Leu Tyr His Ala Ala Arg Lys Leu Glu  
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<211> 61

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<213> Solanum tuberosum

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1260

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1320

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&lt;210&gt; 5

&lt;211&gt; 546

&lt;212&gt; PRT

&lt;213&gt; Solanum tuberosum

&lt;300&gt;

&lt;308&gt; Swiss Prot / P30924

&lt;309&gt; 1993-07-26

&lt;400&gt; 5

Met Lys His Ser Ser Ala Ile Ser Ala Val Leu Thr Asp Asp Asn Ser  
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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 03/14840

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/54 C12N15/82 C12N5/10 A01H5/00 A01H5/10  
A23L1/0522

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00/08184 A (HOECHST SCHERING AGREVO GMBH) 17 February 2000 (2000-02-17) cited in the application	18,27-35
Y	* the whole document; in part. examples 5 and 12 *	1-38
X	SCHWALL GERHARD P ET AL: "Production of very-high-amylose potato starch by inhibition of SBE A and B." NATURE BIOTECHNOLOGY, vol. 18, no. 5, May 2000 (2000-05), pages 551-554, XP002241849 ISSN: 1087-0156 the whole document	18,27, 29-35

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*&\* document member of the same patent family

Date of the actual completion of the international search

18 March 2004

Date of mailing of the international search report

05/04/2004

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NL - 2280 HV Rijswijk  
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Fax: (+31-70) 340-3016

Authorized officer

Kania, T



# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP 03/14840

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>ABEL G J ET AL: "Cloning and functional analysis of a cDNA encoding a novel 139 kDa starch synthase from potato (<i>Solanum tuberosum</i> L.)"</p> <p>PLANT JOURNAL, BLACKWELL SCIENTIFIC PUBLICATIONS, OXFORD, GB, vol. 10, no. 6, December 1996 (1996-12), pages 981-991, XP002121809 ISSN: 0960-7412 cited in the application page 985, right-hand column, paragraph 1 page 986, right-hand column - page 988</p>	18,27, 30-35
X	<p>EDWARDS ET AL: "A combined reduction in activity of starch synthases II and III of potato has novel effects on the starch of tubers"</p> <p>PLANT JOURNAL, BLACKWELL SCIENTIFIC PUBLICATIONS, OXFORD, GB, vol. 17, no. 3, February 1999 (1999-02), pages 251-261, XP002115084 ISSN: 0960-7412 page 254 - page 257, left-hand column</p>	18,27, 29-35
X	<p>WO 99/66050 A (MARTIN CATHERINE ROSEMARY ;SCHWALL GERHARD PETER (GB); SMITH ALISO) 23 December 1999 (1999-12-23) the whole document</p>	18,27, 29-35
X	<p>LLOYD J ET AL: "Simultaneous antisense inhibition of two starch-synthase isoforms in potato tubers leads to accumulation of grossly modified amylopectin"</p> <p>BIOCHEMICAL JOURNAL, PORTLAND PRESS, LONDON, GB, vol. 338, no. PART 2, 1 March 1999 (1999-03-01), pages 515-521, XP002115085 ISSN: 0264-6021 the whole document</p>	18,27, 29-35
X	<p>WO 96/34968 A (GIDLEY MICHAEL JOHN ;WESTCOTT ROGER JOHN (GB); COOKE DAVID (GB); D) 7 November 1996 (1996-11-07) page 27 - page 34</p>	18,27, 28,31-35
Y	<p>WO 97/20040 A (AMYLOGENE HB ;EK BO (SE); KHOSNOODI JAMSHID (SE); LARSSON CLAS TOM) 5 June 1997 (1997-06-05) * the whole document; in part. page 9 line 29 - page 10 line 8 *</p>	1-38
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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 03/14840

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SLATTERY CASEY J ET AL: "Engineering starch for increased quantity and quality." TRENDS IN PLANT SCIENCE, vol. 5, no. 7, July 2000 (2000-07), pages 291-298, XP002241850 ISSN: 1360-1385 the whole document	
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Information on patent family members

International Application No

PCT/EP 03/14840

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